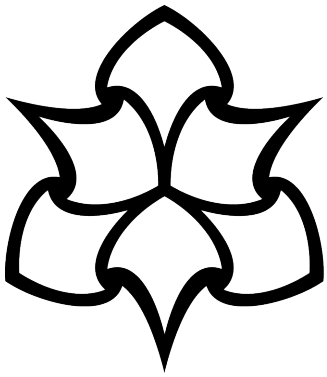

Examining the Role of Novel Lignan Compounds as Anti-Leukaemia Agents

A thesis submitted in fulfilment of the requirements of the
Manchester Metropolitan University
for the degree of Master of Science (by Research)

School of Healthcare Science

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Declaration

The work in this thesis is original and has not been submitted previously in support of any qualification or course.

Signed:

Date:

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Abstract

Leukaemia is a family of haematological malignancies and is responsible for approximately 4500 deaths in the UK annually. Despite extensive research and development within this disease area over the last decade, there has been little change in the overall survival, risk of relapse and side effects, highlighting the need for improved chemotherapeutics.

Lignans are polyphenolic compounds found naturally in plants and have been suggested to have antioxidant properties and inhibitory effects on the Wnt/ß-catenin and the PI3K/Akt cell signalling pathways which are aberrantly activated in leukaemia. A series of eighteen novel compounds have been synthesised based on a natural lignan extract, each with structural modifications to the side chains of each molecule. The effects of the eighteen lignan derivatives on the leukaemia cell line Jurkat were investigated. Biochemical activity as an indirect measure of viability was assessed using the MTS assay, while apoptosis and necrosis were investigated using the Annexin V/Propidium Iodide assay. Cell cycle analysis by DNA quantification was also performed to determine any arrest in cycle induced by the compounds. Additionally, Western Blotting analysis was performed on lignan treated cell lysates to identify changes in protein expression that would suggest effects on the Wnt/ß-catenin and the PI3K/Akt signalling pathways. HSPB1 and HSPA1A expression was analysed to determine the cellular stress response.

Data indicate that the lignan compounds SD007, SD013 and SD018 significantly affect cellular metabolism and induce apoptosis following treatment. Cell cycle analysis demonstrates there is a significant reduction in Jurkat cells entering G_2-phase, which further indicates the pro-apoptotic effect of the lignan compounds. Western Blotting analysis suggests there is a cellular stress response induced by lignan compound treatment, due to the increased expression of the stress protein HSPB1. However, no change in phosphorylated levels of ß-catenin or Akt were observed, suggesting that these particular lignan compounds have no effect on the Wnt/ß-catenin or the PI3K/Akt pathways.

Further study is necessary to establish the cell signalling cascades affected by lignan compound treatment, however the data obtained highlight lignan compounds SD007, SD013 and SD018 for further development and refinement as anti-leukaemia agents.

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List of Abbreviations

17DMAG	17-Dimethylaminoethhylamino-17-demetho**
ALL	Acute Lymphoblastic Leukaemia
APC	Adenomatous Polyposis Coli
ATP11C	ATPase Phospholipid Transporting 11C
BCL	B-cell Lymphoma
BSA	Bovine Serum Albumin
CK1	Casein Kinase 1 α
CLL	Chronic Lymphocytic Leukaemia
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
ECL	Enhanced Chemiluminesence
FADD	Fas-associated protein with death domain
FBS	Foetal Bovine Serum
GSK3-ß	Glycogen Synthase Kinase 3 - ß
HSC	Haematopoietic Stem cell
HSP	Heat Shock Protein
LEF/TCF	Lymphoid Enhancer Factor/T Cell Factor
LRP5/6	Low density Lipoprotein Receptor Like Protein 5/6
MOMP	Mitochondrial Outer Membrane Permeablization
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NOTCH1	Notch homolog 1, translocation-associated (Drosophilia)
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PI	Propidium Iodide
PS	Phosphatidylserine
RIPA	Radioimmunoprecipitation Buffer
ROS	Reactive Oxygen Species
T-ALL	Acute T-cell Lymphoblastic Leukaemia
TNF	Tumor Necrosis Factor
TP53	Tumor Protein p53

1.0 Introduction

1.1 Background to leukaemia and treatments

Leukaemia is the collective term for a family of haematological malignancies that are characterised by the aberrant proliferation of immature leukocytes initially in the bone marrow. There are in excess of 9000 cases of leukaemia diagnosed each year in the UK, with approximately 4500 deaths attributed to the disease annually (Neal and Hoskin, 2012). It is also the most abundant form of paediatric cancer (ages 0-14), accounting for 30% of cancer diagnoses, 78% of which can be classified as Acute Lymphoblastic Leukaemia (ALL; Cancer Research UK, 2017). The 5-year survival rate for patients with ALL is 70%, however this varies depending on factors such as age, subtype, cytogenetic aberrations and responsiveness to treatment. (Cancer Research UK, 2017).

Broadly, the subclassification of leukaemia is determined by categorising the disease onset as either acute or chronic, and the malignant cell as belonging to either the myeloid or lymphoid cell lineage (Neal and Hoskin, 2012). Specifically, acute T-cell lymphoblastic leukaemia (T-ALL) is a malignancy which occurs during the lymphoblast stage of haematopoiesis (Figure 1.1.1), which affects the clonal production of T-lymphocytes. T-ALL accounts for between 15-20% of ALL diagnoses and is associated with a poor prognostic outcome (Hoelzer and Gökbuget, 2009).

Deregulated proliferation of the immature lymphoblasts within the bone marrow suppresses the production of erythrocytes, thrombocytes and leukocytes (Neal and Hoskin, 2012). This failure of the bone marrow instigates the typical symptoms of leukaemia, which includes anaemia, frequent and unexplained bleeding, and susceptibility to infections. Additionally, swelling of the liver, spleen and lymph nodes can occur due to extramedullary haematopoiesis in these organs following bone marrow failure (Kim, 2010).

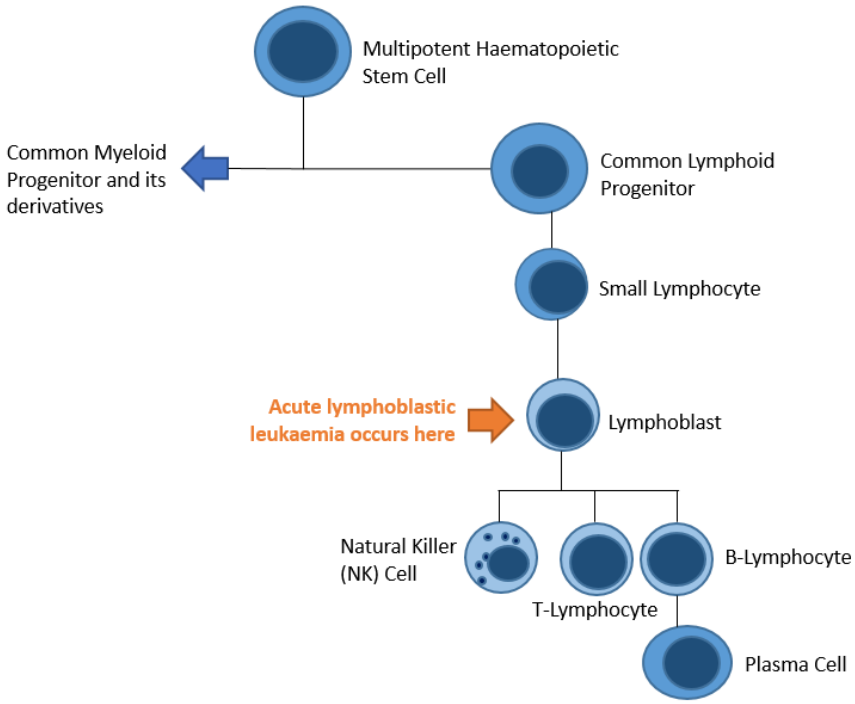


Figure 1.1.1: Haematopoietic stem cell tree, highlighting the lymphoid lineage adapted from Cancer Research UK.

Chromosomal abnormalities or gene mutations within the haematopoietic stem cell are an established characteristic of leukaemia, frequent examples of which include mutations affecting tumour protein 53 (TP53) and Notch Homolog 1, translocation associated (Drosophilia; NOTCH1), transcription factor rearrangements, and the presence of the Philadelphia chromosome (Zuckerman and Rowe, 2014). While these chromosomal aberrations may be pathogenetically significant, in T-ALL these are not reason for subclassification due to varying biological features among patients (Vardiman et al., 2009). The prognosis and treatment options can vary depending on the karyotype of the patient, with some chromosomal abnormalities responding positively with targeted therapies (An et al., 2010). An example of this is using dasatinib or imatinib as specific agents in Philadelphia chromosome positive T-ALL patients.

Typically, treatment for ALL comprises anti-cancer chemotherapy, and is received in three stages – induction, consolidation and maintenance (Neal and Hoskin, 2012). Options for induction chemotherapy include varying combinations of daunorubicin, vincristine, prednisone, asparaginase and occasionally cyclophosphamide (Bailey, 1992; Joel and Rohatiner, 2002). Use of these drugs is accompanied by a wide range

of side effects due to the non-specific nature of the drug for the T-ALL cell. Once the patient has achieved remission, consolidation treatment will be started to prevent re-emergence of the leukaemic clone. Drugs used in this phase include cytarabine (Ara-C), etoposide, methotrexate and 6-mercaptopurine, all of which are also targeting any rapidly dividing cells. Maintenance therapy consists of lower dose chemotherapy with the same drugs for approximately 18-24 months to achieve long-term remission. Due to the non-specific nature of anti-cancer chemotherapy, debilitating side effects of the treatment such as fatigue, nausea and a weakened immune system are common. Additionally, despite achieving a remission, some patients can become refractory to the drugs used during treatment. Patients with Philadelphia chromosome positive chronic myeloid leukaemia (CML) may become resistant to imatinib mesylate, which would require an alternative tyrosine kinase inhibitor or other treatment to be sought (Bitencourt et al., 2011).

Side effects, along with acquired and inherited resistance to the aging chemotherapy arsenal, makes continued research into newer targeted agents for the disease a necessity (Alfarouk et al., 2015; Neal and Hoskin, 2012). Current clinical trials such as the UKALL 14 which focuses on adults with ALL, and UKALL2011 which focuses on patients aged 1-24 years, have been designed to improve our understanding of how modifications to the current protocols can both sustain disease remission and minimise side effects (Vora, 2012; Neal and Hoskin, 2012).

1.2 Cytotoxicity and Cellular Stress

1.2.1 Cell Death Machinery – Apoptosis and necrosis

Cell death is a natural physiological process that occurs in the life of all organisms, and can be distinguished by two alternative modes, apoptosis and necrosis (Adamczyk et al., 1998).

Apoptosis, which is also known as programmed cell death, is a vital part of normal cell turnover and development. Approximately ten billion cells are apoptosed daily in order to maintain cell number homeostasis (Renehan et al., 2001). Evasion of apoptosis is one of the six hallmarks of cancer (Hanahan and Weinberg, 2011), which in some patients with chronic lymphocytic leukaemia (CLL) for example, could be attributed to a combined over-expression of the bcl2 oncogene and dysregulation of

the tumour suppressor gene p53 (Otake et al., 2007). Morphologically, apoptosis is characterised by cell shrinkage and chromatin condensation, events that follow the early translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane (Elmore, 2007). Cysteine-aspartic proteases (caspases) are enzymes essential to the apoptotic process, that are activated by either the extrinsic (death receptor) or the intrinsic (mitochondrial) apoptotic signalling pathways (Ichim et al., 2016). Both pathways converge at the same point to form the execution phase (Elmore, 2007).

The extrinsic pathway (Figure 1.2.1) is activated by the binding of a ligand to the corresponding death receptor, such as tumour necrosis factor (TNF)-related apoptosis-inducing ligand receptor 1 (TRAILR1) or first apoptosis signal (FAS) receptor (Ichim et al., 2016). Fas-associated protein with death domain (FADD), an adapter protein, heterodimerises pro-caspase-8 and pro-caspase-10, which activates the initiators caspase-8 and caspase-10. The execution pathway begins, where caspase-8 cleaves and activates caspase-3 and caspase-7. Caspase-3 inactivation of the flippase ATPase Phospholipid Transporting 11C (ATP11C) and activation of scramblase XK-related protein 8 (Xkr8) is one of the mechanisms responsible for the externalisation of PS, which facilitates phagocytosis of the apoptotic cell by macrophages (Segawa and Nagata, 2015).

The intrinsic pathway (Figure 1.2.2) is a mitochondria-based apoptosis mechanism, which is initiated by a variety of lethal stimuli including DNA damage, hypoxia and metabolic stress (Ichim et al., 2016). These stimuli lead to the activation of pro-apoptotic effectors Bax and Bak, which results in mitochondrial outer membrane permeabilisation (MOMP) and the ensuing release of the pro-apoptotic protein cytochrome c into the cytosol (Brentnall et al., 2013). Cytochrome c associates with apoptotic protease activating factor 1 (Apaf-1) to form the apoptosome, which activates caspase-9. Caspase-9 cleaves and activates caspase-3 and caspase-7, and as with the extrinsic pathway, the execution phase continues (Elmore, 2007).

Cross talk between the extrinsic and intrinsic pathways occur through caspase-8 cleavage of the BH3-only protein Bid, generating the active truncated form of Bid (tBid) (Brentnall et al., 2013). tBid also activates Bax and Bak, thus resulting in MOMP

and cytochrome c release, serving to further amplify the apoptotic process (Ichim et al., 2016).

The anti-apoptotic protein Bcl-2 exists to block the further activation of Bax, Bak and Bid, which prevents MOMP from occurring (Ichim et al., 2016). In normal cells, Bcl-2 is essential for development and sustaining cellular homeostasis, however, as previously mentioned, deregulation of Bcl-2 expression in malignancy can contribute to the prevention of apoptosis in cancer cells (Otake et al., 2007).

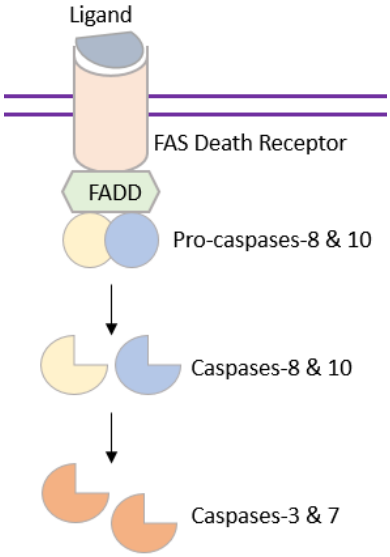


Figure 1.2.1: Extrinsic apoptosis pathway adapted from Elmore et al. 2007

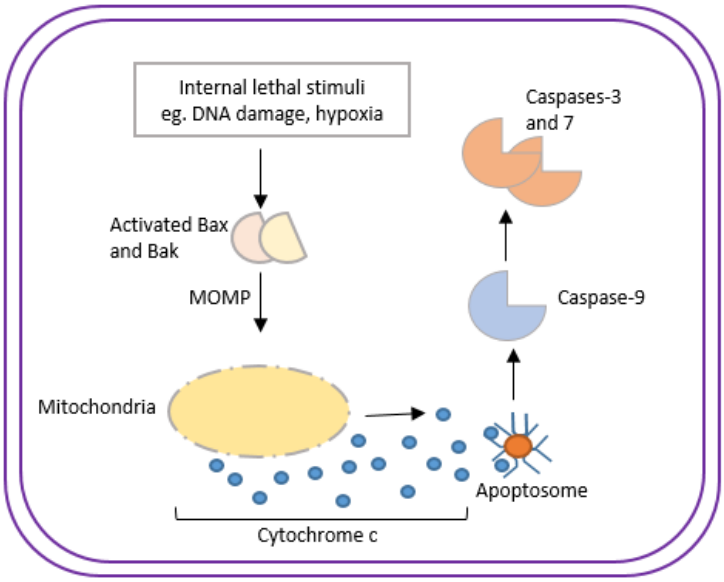


Figure 1.2.2: Intrinsic apoptosis pathway adapted from Elmore et al. 2007

Necrosis can be differentiated from apoptosis by changes in cell morphology (Figure 1.2.3), which includes swelling of the cell and its organelles, production of reactive oxygen species (ROS) and irreversible loss of plasma membrane integrity (Matsumura et al., 2000). Necrosis can occur as a result of a number of cellular stresses, including hypoxia, complement attack, and toxin exposure (Matsumura et al., 2000). Ligands belonging to the tumour necrosis factor (TNF) family are known initiators of both apoptosis and necrosis, the outcome of which is determined by both the type of cell and the particular context (Festjens et al., 2006). For example, Fas binding in the absence or deficiency of FADD will not lead to caspase-8 activation and therefore the extrinsic apoptotic pathway cannot proceed, so the cell will induce necrosis (Festjens et al., 2006).

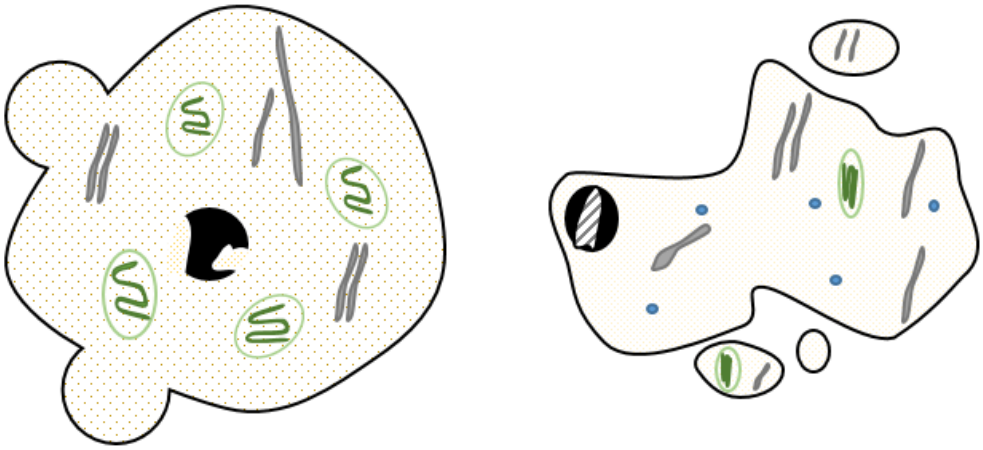


Figure 1.2.3: Differences in cell morphology during the processes of necrosis (left) and apoptosis (right) - adapted from Ichim et al. 2016.

Despite the reputation of necrosis as being an unorganised process, relatively recent literature has suggested that necrosis is another form of programmed cell death (Vanden Berghe et al., 2013; Edinger and Thompson, 2004). However, necrosis differs from apoptosis by the activation of a local inflammatory response, which is triggered by the release of cytokines from the necrotic cell (Golstein and Kroemer, 2007). The inflammatory response can be beneficial to the host by alerting the immune system to cell death and initiating tissue repair, however inflammation can elicit damage of host tissues by ROS and proteases, which serve to enhance the

pathogenesis of the underlying disease and exacerbate the condition of the host (Rock and Kono, 2008).

1.2.2 Heat Shock Proteins and Cellular Stress

Heat Shock Proteins (HSPs) are a group of molecular chaperones that under normal conditions, facilitate and sustain protein folding, as well as assist with protein trafficking (Kaul and Thippeswamy, 2011). A number of HSPs are upregulated during stressful conditions to prevent protein unfolding and inhibit cell death pathways, and once the stress stimulus passes, the levels of HSPs return to a baseline level (Dempsey-Hibbert et al., 2012). Transcription is regulated by transcription factors from the heat shock factor (HSF) family, which ensures swift activation and subsequent deactivation following recovery from the stressful stimuli (Ciocca and Calderwood, 2005).

HSPs were previously categorised into families based on the molecular weights of the proteins themselves. However, following the discovery of more HSPs and the sequencing of the human genome, HSP nomenclature was reviewed and the proteins were renamed after the genes responsible for their synthesis (Kampinga et al., 2009).

A wealth of studies have documented the role of HSPs in human cancers (Ciocca and Calderwood, 2005), especially the contribution of HSPB1, HSPA1A and HSPC1 (previously known as Hsp27, Hsp72 and Hsp90, respectively).

HSPB1 and HSPA1A have been associated with conferring anti-apoptotic properties to cancer cells where they are over-expressed (Dempsey-Hibbert et al., 2012). The transfection of a plasmid containing HSPB1 into testicular cancer cells conferred resistance to the chemotherapeutic agents cisplatin and doxorubicin (Richards et al., 1996). HSPA1A has been shown to inhibit the formation of the apoptosome by directly targeting Apaf-1 (Kuwana et al., 2000). However conflicting data do exist, and HSPA1A has also been associated with increased Fas-mediated apoptosis in leukaemic T-cells (Creagh et al., 2000).

Increased levels of HSPs in leukaemia patients have been correlated with a poor prognosis (Dempsey-Hibbert et al., 2012). This is not universal in all cancers, as HSPA1A expression in osteosarcoma is associated with higher sensitivity to treatment, but HSPB1 expression is still associated with a poor prognosis (Ciocca and Calderwood, 2005). However, as observed in CLL, over-expression of one HSP does not suggest that levels of other HSPs may be elevated simultaneously (Dempsey et al., 2010).

Previous literature has suggested that existing elevated levels of several families of HSPs are associated with chemotherapy resistance in some cancers. For example, elevated levels of HSPB1 has been associated with cisplatin resistance in cervical cancer (Zhu et al., 2016). Furthermore, a cytotoxic agent can induce the expression of several HSPs from baseline levels, which itself can confer resistance against the agent. Attention has been drawn to the combined use of HSP inhibitors and chemotherapeutic agents, with the aim of preventing drug-induced HSP expression and consequent drug resistance (Sulthana et al., 2017; Li et al., 2009). HSPC1 (also known as Hsp90) inhibitors, such as AUY922 and 17-DMAG in particular, have shown moderate success in Phase I and Phase II clinical trials, and more recently HSPB1 has been targeted using an anti-sense inhibitor known as apatorsen (OGX-427) (Oki et al., 2015; Mellatyar et al., 2016; Chi et al., 2016). It is important to understand the effects of any prospective new drug compound on the heat shock response to be able to foresee the potential for resistance to the compound.

1.3 Cell Signalling

1.3.1 Wnt/β-Catenin Pathway

The canonical Wnt/β-Catenin pathway (Figure 1.3.1.1) is an evolutionarily conserved cell-signalling cascade that is essential from the earliest stages of mammalian embryonic development in virtually all tissues and continues throughout the life of an organism (Kahn, 2014). There are nineteen different Wnt ligands within the Wnt family, and ten receptors for the ligands to associate, which lends to the overall complexity of the cascade (Lento et al., 2013).

The pathway is responsible for regulating proliferation and development of the haematopoietic stem cell (HSC), ensuring the balance between self-renewal and

differentiation is maintained (Ashihara et al., 2015). The signalling cascade is active in the developing T-cell until single positive selection in the thymus, at which point the levels of the Wnt ligand are negligible (Gopal et al., 2014). In haematological malignancy, the pathway is aberrantly activated, which contributes to the characteristic uncontrolled proliferation of immature blast cells (Ashihara et al., 2015).

β-catenin is a multifunctional protein found in a number of subcellular compartments and is essential in the formation of the cytoskeleton. In the pathways "off" state, the β-catenin destruction complex, which consists of adenomatous polyposis coli (APC), the scaffold protein Axin, glycogen synthase kinase 3-β (GSK3β) and casein kinase 1α (CK1), ubiquitinates β-catenin which targets the protein for degradation by the proteasome (Ashihara et al., 2015). However, in the "on" state, the Wnt ligand binds to frizzled (Fz) and its co-receptor lipoprotein receptor-related protein 5/6 (LRP5/6). This leads to the binding of Dishevelled (Dvl) with Fz, which interrupts the formation of the β-catenin destruction complex, which in turn allows β-catenin to escape degradation and accumulate in the cytoplasm (Ashihara et al., 2015; Bafico et al., 2004; MacDonald et al., 2009). The β-catenin enters the nucleus and binds to lymphoid enhancer factor/T-cell factor (LEF/TCF), which allows transcription of target genes such as cyclin D1 and c-myc and drives cell proliferation (Pećina-Šlaus, 2010).

The role of the pathway in proliferation has highlighted the opportunity for therapeutic intervention. Over the past few years, pathway inhibitors have been developed and entered Phase I clinical trials with some success (Lu et al., 2016). However, due to the role of the Wnt/β-catenin pathway in normal homeostasis and other pathway interactions, it is important that novel anti-cancer targeted treatments do not affect the regular tissue development and repair (Kahn, 2014; Zhang and Hao, 2015).

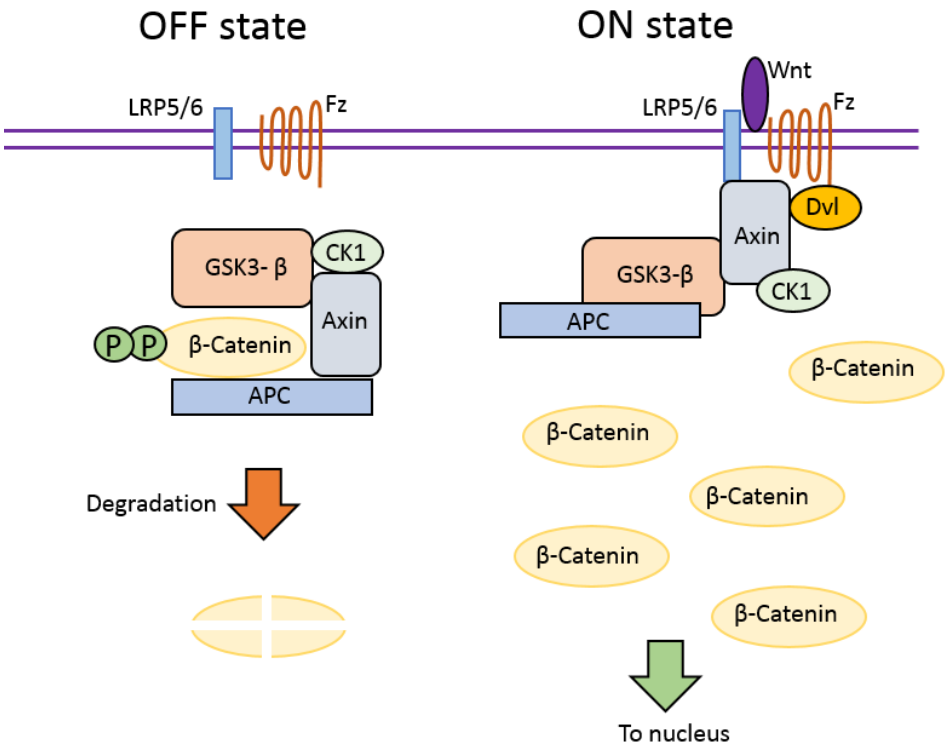


Figure 1.3.1: The canonical Wnt/β-catenin pathway as occurs in both the presence and absence of Wnt – adapted from Ashihara et al. 2015.

1.3.2 PI3K/Akt Pathway

The PI3K/Akt pathway is a complex cell signalling cascade involving many components that promotes cell survival and growth and is also important in the regulation of the cell cycle (Fekete et al., 2012).

Binding of extracellular factors to receptor tyrosine kinases (RTK) phosphorylates the receptor and leads to the recruitment and activation of phosphoinositide-3-kinase (PI3K). The activated PI3K phosphorylates intracellular membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), which leads to the recruitment and activation of the proto-onco protein Akt (also known as PKB) (Nicholson and Anderson, 2002). In addition to other subcellular functions which remain to be elucidated, Akt is able to suppress MOMP by phosphorylating pro-apoptotic Bad and thus promoting cell survival (Song et al., 2005).

Literature has suggested that the PI3K/Akt pathway can accelerate cell cycle progression, a potential mechanism of which is the over-expression of cyclin D1 as a result of upregulation by Akt (Nicholson and Anderson, 2002; Chang et al., 2003).

Targeted therapies affecting the PI3K/Akt pathway have been developed, such as KRX-0401 and GSK263677. and are undergoing Phase I and Phase II clinical trials with varying degrees of success in colorectal cancer patients (Bahrami et al., 2017).

1.4 Lignans

Lignans are a family of diphenolic plant compounds, which are particularly abundant in flax and sesame seeds (Ramos, 2008). They are also present in some fruits, vegetables and grains, albeit at a lower concentration (Peterson et al., 2010). The basic chemical structure of a lignan consists of two C_6C_3 units (propylbenzene; Figure 1.4.1), which are linked by a carbon-carbon bond at position 8,8' (Moss, 2000). Previous literature has indicated that natural lignans possess anti-cancer, anti-oxidant, and anti-inflammatory properties (Saleem et al., 2005; Peterson et al., 2010), which has led to a heightened interest in their pharmacological potential.

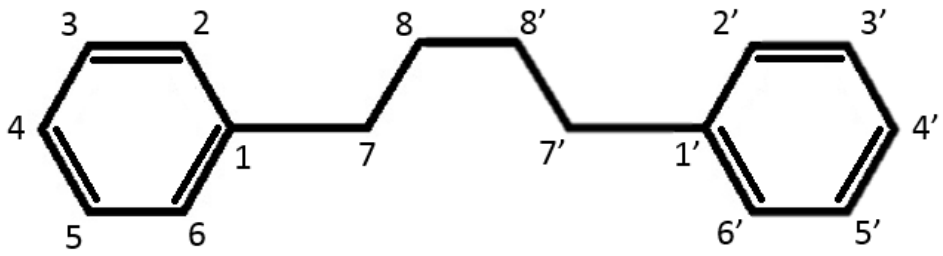


Figure 1.4.1: Chemical structure of a lignan, coupled at the 8,8' position - adapted from Moss, 2000.

The metabolism of plant lignans by intestinal bacteria produces the mammalian lignans enterodiol and enterolactone, the latter of which has also been identified as a useful biomarker for measuring dietary lignan exposure (Kuijsten et al., 2005). Enterodiol and enterolactone possess oestrogen-like biological properties and can therefore bind to the type II oestrogen receptor in place of oestradiol. Depending on the dose and cancer cell line, this can induce either oestrogenic or antioestrogenic responses (Wang, 2002). This is promising for further research in the treatment of hormone-sensitive cancers, such as those of the breast and prostate.

The cytotoxic and cytostatic properties of lignan-based compounds have been previously described in the literature. The lignan podophyllotoxin is too cytotoxic to

be considered for cancer treatment, however its semi-synthetic derivatives, etoposide and teniposide, are included in treatment protocols for some cancers, including leukaemia and lymphoma (Gordaliza et al., 2000). Etoposide and teniposide, which are cytostatic agents, cause breaks in DNA by inhibiting topoisomerase IIa, which subsequently arrests the cancer cells in G_2/M phase. Despite repair of the DNA breaks, affected cells lose their clonogenicity (Schonn et al., 2010). As teniposide is more readily absorbed by cells, accumulation within the cells can result in cytotoxicity (Long, 1992).

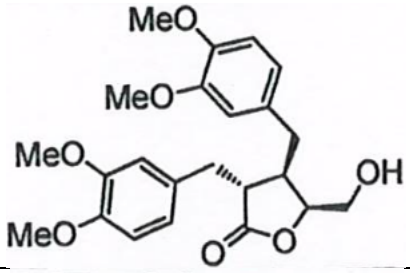
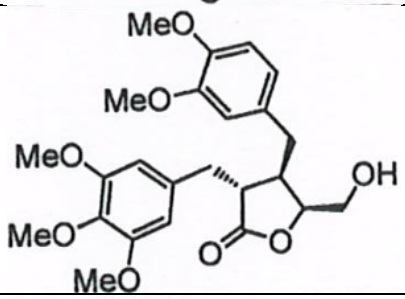
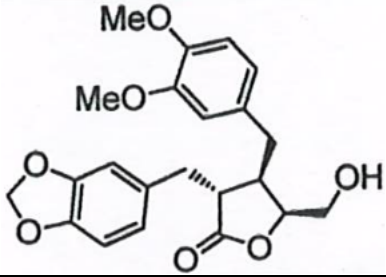
Viability assays determined that leukaemia and melanoma cell lines were more sensitive to treatment with the natural lignan (-)-sesamin, than brain and ovarian cancer cell lines (Saeed et al., 2014). The same study also compared the IC_{50} values of (-)-sesamin with those of other lignans and derivatives, including arctigenin, podophyllotoxin, teniposide and etoposide. This comparison demonstrated that despite (-)-sesamin and arctigenin being less cytotoxic than podophyllotoxin, etoposide and teniposide, they are still promising candidates for further cancer drug development (Saeed et al., 2014).

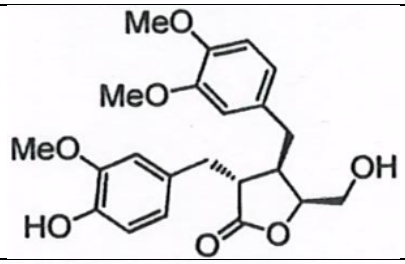
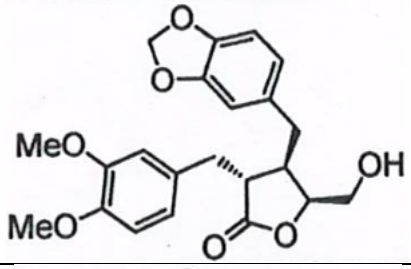
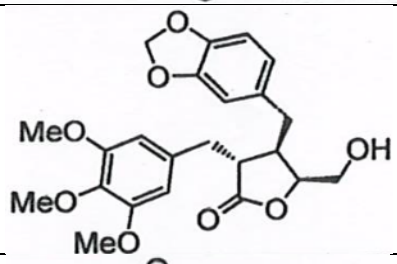
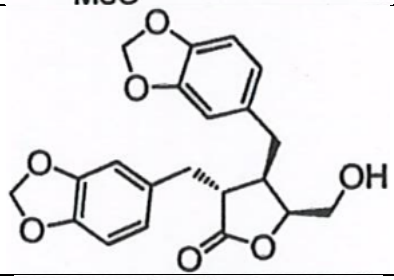
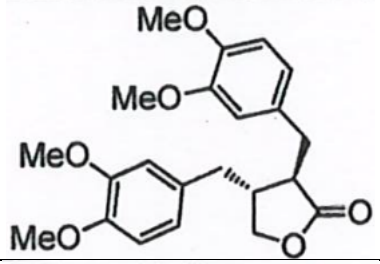
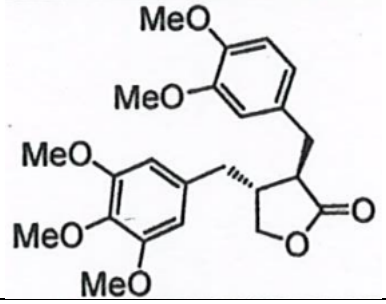
Adding to data showing cytostatic properties of lignans, studies have also shown that lignans possess pro-apoptotic properties. The naturally-derived lignan arctigenin upregulated the expression of the pro-apoptotic gene Bad in a T-cell lymphoma cell line (Su et al., 2015), and apoptosis was also observed following treatment of colon carcinoma cells with arctigenin (Hausott et al., 2003). Another study showed that expression of the pro-apoptotic protein Bax was upregulated and the expression of anti-apoptotic Bcl-2 was downregulated in fourteen different cancer cell lines following treatment with the novel synthetic lignan Vitexin 6 (Zhou et al., 2013).

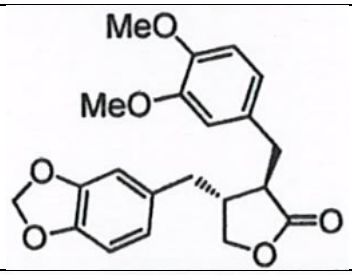
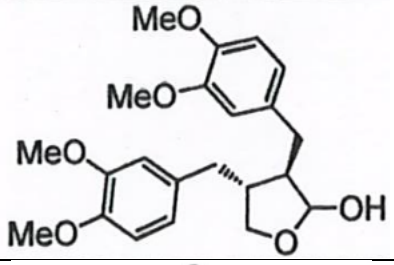
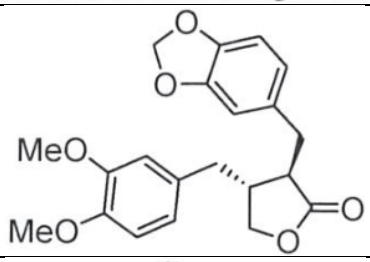
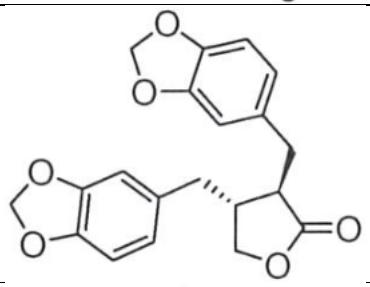
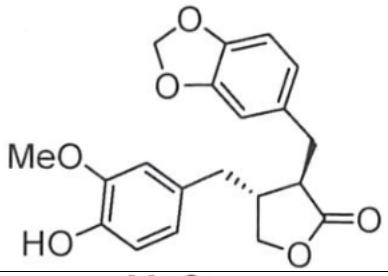
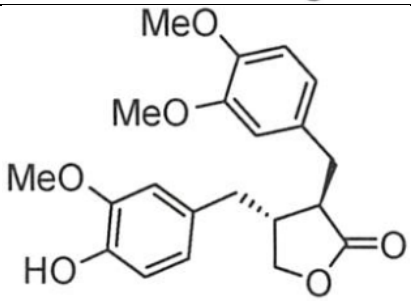
A previous study showed that the treatment of a colon cancer cell line (SW480) with three different lignans reduced the levels of β-catenin, with arctigenin showing the most significant effect (Yoo et al., 2010). Additionally, a previous study demonstrated that treatment of a prostate cancer cell line (LNCaP) with the lignan matairesinol, decreased Akt activity which had previously been implicated as responsible for the resistance LNCaP cells to TRAIL-induced apoptosis (Peuhu et al., 2010).

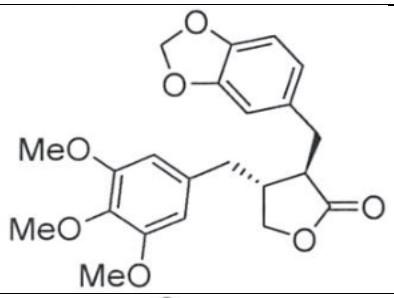
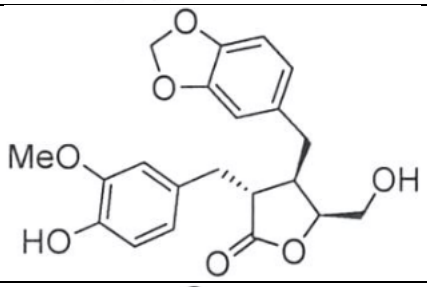
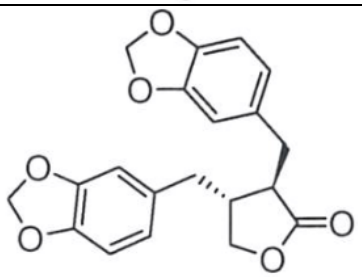
Our collaborators in the School of Chemical Sciences at the University of Auckland, New Zealand, synthesised a bank of eighteen lignan derivatives. These were produced by structural modifications of the original butyrolactone extract, the purpose of which was to have a direct effect on the efficacy of the compound. The eighteen lignan compounds are indicated in Table 1.4.1. No previous anti-cancer research has been performed using this particular group of eighteen novel lignan compounds, emphasising the novelty of this particular study.

Table 1.4.1: Code, chemical structure, and molecular weights of each lignan derivative.

Code	Chemical Structure	Molecular Weight (kDa)
SD001		416.4642
SD002		446.4902
SD003		400.4218

SD004		402.4376
SD005		400.4218
SD006		430.4530
SD007		384.3840
SD008		386.4440
SD009		416.4642

SD010		370.4010
SD011		386.4600
SD012		370.4010
SD013		354.3580
SD014		356.3740
SD015		372.4170

SD016		400.4270
SD017		386.4000
SD018 [(-)-SD013]		354.3580

1.5 Aims and Objectives

It is clear from the previous literature that some members of the lignan family have anti-cancer properties and some seem to show greater efficacy than others. However, their mode of action and effects on the cellular stress response remain to be determined. The aim of the project was to identify the potential of the synthetic lignans as anti-leukaemia agents. This was achieved by accomplishing the following objectives:

1. To determine the cytotoxic/cytostatic effects of a new set of novel lignan compounds on the Jurkat T-cell leukaemia cell line.
2. To elucidate the signalling pathways affected by the compounds.
3. To establish the degree of cellular stress caused by the compounds by analysis of HSP expression.

2.0 Materials and Methods

2.1 Materials

2.1.1 Consumables

0.2 mL PCR Tubes *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *I1402-8100*
0.5 mL Microcentrifuge Tubes *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *S1605-0000*
1.5 mL Microcentrifuge Tubes *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *S1615-5500*
1000 µL XL Graduated Filter Tip *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *S1122-1730-C*
10 µL Graduated Filter Tip *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *S1121-2710-C*
1250 µL XL Graduated Tip *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *S1112-1720-C*
2.0 mL Microcentrifuge Tubes *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *S1620-2700*
200 µL UltraPoint Graduated Tip *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *S1113-1700-C*
200 µL Graduated Filter Tip *Starlab UK Ltd.* *Milton Keynes, Buckinghamshire, UK*	*Product* *S1120-8710-C*
Bijoux Container Tubes (7 mL) *Scientific Laboratory Supplies Ltd.* *Nottingham, Nottinghamshire, UK*	*Product* *SLS7522*
Centrifuge Tube 15 mL *Sarstedt Ltd.* *Leicester, Leicestershire, UK*	*Product* *62.554.502*

Centrifuge Tube 50 mL *Sarstedt Ltd.* *Leicester, Leicestershire, UK*	*Product* *62.547.254*
Falcon® 5 mL Polystyrene Round Bottomed Tubes *Corning Ltd.* *Deeside, Flintshire, UK*	*Product* *352052*
Nunc™ 25 cm^2 Cell Culture Flasks *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *156367*
Nunc™ Microwell™ 12 Well Microplates *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *150628*
Nunc™ Microwell™ 24 Well Microplates *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *142475*
Nunc™ Microwell™ 48 Well Microplates *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *150687*
Nunc™ Microwell™ 96 Well Microplates *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *167008*
Serological Pipette 10 mL *Sarstedt Ltd.* *Leicester, Leicestershire, UK*	*Product* *86.1254.001*
Serological Pipette 25 mL *Sarstedt Ltd.* *Leicester, Leicestershire, UK*	*Product* *86.1685.001*
Serological Pipette 5 mL *Sarstedt Ltd.* *Leicester, Leicestershire, UK*	*Product* *86.1253.001*
Universal Container Tubes (30 mL) *Scientific Laboratory Supplies Ltd.* *Nottingham, Nottinghamshire, UK*	*Product* *SLS7500*

2.1.2 Reagents

10X Annexin V Binding Buffer *BD Biosciences* *Oxford, Oxfordshire, UK*	*Product* *556454*
30% Acrylamide/Bis *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Product* *161-0158*
(S)-(+)-Camptothecin *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *C9911*
Akt Antibody *Cell Signalling Technology, Inc.* *Leiden, The Netherlands*	*Product* *9272S*
Ammonium Persulphate *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *A3678*
Annexin V-FITC *BD Biosciences* *Oxford, Oxfordshire, UK*	*Product* *556419*
Anti-Rabbit IgG, HRP-linked Antibody *Cell Signalling Technology, Inc.* *Leiden, The Netherlands*	*Product* *7074S*
β-Actin (13E5) Rabbit mAb *Cell Signalling Technology, Inc.* *Leiden, The Netherlands*	*Product* *4970S*
Bovine Serum Albumin Fraction V *Roche Products Ltd.* *Welwyn, Hertfordshire, UK*	*Product* *10735086001*
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay *Promega UK* *Southampton, Hampshire, UK*	*Product* *G3580*
CS&T Research Beads *BD Biosciences* *Oxford, Oxfordshire, UK*	*Product* *650621*
Dimethyl Sulphoxide (DMSO) Fisher Bioreagents UK Ltd. *Loughborough, Leicestershire, UK*	*Product* *BP231*

Dulbecco's Phosphate-Buffered Saline *Lonza Biologics Plc* *Slough, Berkshire, UK*	*Product* *BE17-512F*
Ethanol, Absolute (200 Proof) *Fisher Scientific UK Ltd.* *Loughborough, Leicestershire, UK*	*CAS* *64-17-5*
FACSclean *BD Biosciences* *Oxford, Oxfordshire, UK*	*Product* *340345*
FACSflow *BD Biosciences* *Oxford, Oxfordshire, UK*	*Product* *342003*
Foetal Bovine Serum (Gibco) *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *10270-106*
Glycine *Fisher Scientific UK Ltd* *Loughborough, Leicestershire, UK*	*Product* *BP381-1*
Goat Anti-Mouse IgG (HRP Conjugate) *Enzo Life Sciences (UK) Ltd.* *Exeter, Devon, UK*	*Product* *BML-SA204*
Hsp27 Monoclonal Antibody (G3.1) *Enzo Life Sciences (UK) Ltd.* *Exeter, Devon, UK*	*Product* *ADI-SPA-800D*
Hsp72 Monoclonal Antibody (C92F3A-5) *Enzo Life Sciences* *Exeter, Devon, UK*	*Product* *ADI-SPA-810D*
iScript™ cDNA Synthesis Kit *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Product* *170-8891*
Jurkat, Clone E6.1 *LGC Standards S.a.r.l.* *Teddington, Middlesex, UK*	*Product* *ATCC TIB-152*
N, N, N', N'-Tetramethylethylenediamine (TEMED) *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Product* *161-0800*

NuPage™ LDS Sample Buffer (4X) *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *NP007*
Phosphatase Inhibitor Cocktail II *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *P5726*
Phospho-Akt (Ser473) Antibody *Cell Signalling Technology, Inc.* *Leiden, The Netherlands*	*Product* *9271S*
Phospho-β-Catenin (Ser45) Antibody *Cell Signalling Technology, Inc.* *Leiden, The Netherlands*	*Product* *9564S*
Pierce™ BCA Protein Assay Kit *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *23227*
Pierce™ ECL Western Blotting Substrate *Thermo Fisher Scientific* *Loughborough, Leicestershire, UK*	*Product* *32209*
Precision Plus Protein™ Dual Colour Standards *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Product* *161-0374*
Propidium Iodide *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *P4170*
Protease Inhibitor Cocktail *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *P8340*
RNase A *Roche Products Ltd.* *Welwyn, Hertfordshire, UK*	*Product* *10109142001*
RPMI 1640 Media *Lonza Biologics Plc* *Slough, Berkshire, UK*	*Product* *BE12-115F*
Sodium Chloride *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *S3014*

Sodium Deoxycholate *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *S1827*
Sodium Dodecyl Sulphate *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *L3771*
Trans-Blot® Turbo™ RTA Mini Nitrocellulose Transfer Kit *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Product* *170-4270*
Tris Base *Fisher Scientific UK Ltd* *Loughborough, Leicestershire, UK*	*Product* *BP152-1*
Triton X-100 *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *T8787*
Trypan Blue Solution *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *T8154*
Tween® 20 *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Product* *93773*

2.1.3 Equipment

Centrifuge *Sigma-Aldrich Company Ltd.* *Gillingham, Dorset, UK*	*Model* *3-16-PK*
ChemiDoc™ Touch Imaging System *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Model* *1700-1401*
Class II Safety Cabinet *Labcaire Systems Ltd.* *Southend-on-Sea, Essex, UK*	*Model* *S18R-UV*
CO_2 Incubator *Nuaire Ltd.* *Doncaster, South Yorkshire, UK*	*Model* *NU5500E*

FACSCalibur™ Flow Cytometer *BD Biosciences* *Oxford, Oxfordshire, UK*	*Product* *342973*
FACSVerse™ Flow Cytometer *BD Biosciences* *Oxford, Oxfordshire, UK*	*Product* *651154*
Haemocytometer *VWR International Ltd.* *Lutterworth, Leicestershire, UK*	*Product* *8110204*
Microplate Reader *Bio-Tek® Instruments Ltd.* *Swindon, Wiltshire, UK*	*Model* *Synergy HT*
Microscope *Nikon Corporation Ltd.* *Kingston-upon-Thames, Surrey, UK*	*Model* *TMS-F-301547*
Mini Protean® Tetra Cell, 1.0 mm Plates, Stand, Clamps *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Product* *165-8003FC*
PowerPac™ Basic Power Supply *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Product* *164-5050*
Trans-Blot® Turbo™ Transfer System *Bio-Rad Laboratories Ltd.* *Watford, Hertfordshire, UK*	*Product* *170-4155*

2.1.4 Software

CellQuest Pro *BD Biosciences* *Oxford, Oxfordshire, UK*	*Version* *3.1.1*
FACSuite *BD Biosciences* *Oxford, Oxfordshire, UK*	*Version* *21.0.1*
Gen 5 Data Analysis *Bio-Tek® Instruments Ltd.* *Swindon, Wiltshire, UK*	*Version* *2.0.1*

GraphPad PRISM®	*Version*
GraphPad Software Inc.	*5.0a*
La Jolla, California, USA	
Imagelab	*Version*
Bio-Rad Laboratories Ltd.	*5.2.1*
Watford, Hertfordshire, UK	
Microsoft Excel 2013	*Version*
Microsoft Ltd.	*15.0.4*
Reading, Berkshire, UK	
ModFit LT	*Version*
Verity House Software	*5.0.9*
Topsham, Maine, USA	

2.1.5 Buffers and Solutions

1.0 M Tris-HCl 6.8	***100 mL***
Tris Base	*12.1 g*
dH_2O	*100 mL*
Adjust to pH 6.8	
1.5 M Tris-HCl 8.8	***100 mL***
Tris Base	*12.1 g*
dH_2O	*100 mL*
Adjust to pH 8.8	
1:1000 Primary Antibody	***3 mL***
2.5% BSA in TBS-Tween	*3 mL*
Antibody required	*3 µL*
1:5000 Secondary Antibody	***20 mL***
2.5% BSA in TBS-Tween	*20 mL*
Antibody Required	*4 µL*
10% Ammonium Persulphate	***1 mL***
Ammonium Persulphate	*0.1 g*
dH_2O	*1 mL*
10% Sodium Dodecyl Sulphate	***100 mL***
Sodium Dodecyl Sulphate	*10 g*
dH_2O	*100 mL*

10X Tris Buffered Saline (TBS)	***1 L***
Tris Base	*24 g*
Sodium Chloride	*88 g*
dH_2O	*1 L*
Adjust pH to 7.6	
To make 1X – add 100 mL 10X TBS to 900 mL dH_2O	

1000X Propidium Iodide (PI)	***500* µL**
Propidium Iodide	*25 mg*
DPBS	*500 µL*
To make 1X – add 20 µL to 19.98 mL DPBS	

10X Running Buffer	***1 L***
Tris Base	*30.3 g*
Glycine	*142.6 g*
Sodium Dodecyl Sulphate	*10 g*
dH_2O	*1 L*
To make 1X – add 100 mL 10X Running Buffer to 900 mL dH_2O	

2.5% BSA in TBS-Tween	***20 mL***
BSA Fraction V	*0.5 g*
TBS-Tween	*20 mL*

20X RNase-A	***12.5 mL***
RNase-A	*25 mg*
dH_2O	*12.5 mL*
To make 1X – add 500µL 20X RNase-A to 10mL dH_2O	

5% BSA in TBS-Tween	***20 mL***
BSA Fraction V	*1.0 g*
TBS-Tween	*20 mL*

Resolving Gel (10%)	***15 mL***
dH_2O	*5.9 mL*
30% Bis Acrylamide	*5 mL*
1.5 M Tris 8.8	*3.8 mL*
10% SDS	*150 µL*
10% APS	*150 µL*
TEMED	*6 µL*

Resolving Gel (8%)	***15 mL***
dH_2O	*6.9 mL*
30% Bis Acrylamide	*3.9 mL*
1.5 M Tris 8.8	*3.8 mL*
10% SDS	*150 µL*
10% APS	*150 µL*
TEMED	*8 µL*

RIPA Buffer	***100 mL***
Tris Base	*0.61 g*
Sodium Chloride	*0.29 g*
Sodium Deoxycholate	*0.5 g*
Sodium Dodecyl Sulphate	*0.1 g*
Triton X-100	*1 mL*
dH_2O	*99 mL*
Adjust to pH 7.4	

Stacking Gel (4%)	***6 mL***
dH_2O	*4.1 mL*
30% Bis Acrylamide	*1.0 mL*
1.0 M Tris 6.8	*750 µL*
10% SDS	*60 µL*
10% APS	*60 µL*
TEMED	*6 µL*

TBS with Tween® 20 (TBS-Tween)	***1 L***
1X TBS	*999 mL*
Tween® 20	*1 mL*

2.2 Methods

2.2.1 Compound Reconstitution and Preparation

The lignan compounds were transported in a lyophilized state from the University of Auckland, New Zealand and were a kind gift from Dr. David Barker. Depending on the mass of the compound, they were diluted to concentrations of 5-12 mg/mL using 100% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, UK) as the vehicle. Each compound was vortexed for 5 seconds and placed in a sonicating waterbath for 5 minutes at room temperature. The compounds were then stored at room temperature.

When preparing solutions from stock, the reconstituted lignan compound was diluted in required volumes of RPMI 1640 media (Lonza, UK) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, UK). Serial dilutions were performed from this solution to obtain any other desired concentrations. All lignan compound solutions were prepared at 2X concentration and were therefore diluted to 1X concentration when incubated with the cell suspension at a 1:1 ratio.

2.2.2 Cell Culture

Jurkat E6.1 cells were cultured in RPMI 1640 media supplemented with 10% FBS (herein referred to as 10% RPMI) in vented T25 flasks. Cells were incubated at 37 °C in a 5% CO_2 humidified environment and were routinely sub-cultured upon reaching a density of between 2.0-2.5x10^6 cells/mL.

Before treatment with the compounds, cell viability was assessed using the trypan blue exclusion method. For this, a 100 µL aliquot was taken from the flask and placed in a 0.5 mL microcentrifuge tube and mixed with 100 µL 0.4% trypan blue solution (Sigma, UK). The cell and trypan blue mixture was placed in a haemocytometer and counted under the microscope, with the average number of cells used to calculate the cell density within 1 mL of culture. Trypan blue is excluded from viable cells by the intact cell membrane, while non-viable cells absorb the stain and appear blue under the microscope. Experiments were only performed when cell viability was more than 95% within the given culture of Jurkat cells.

The volume of culture media containing the correct number of cells required for the entire experiment was removed from the suspension and placed in a 15mL centrifuge tube and spun in a centrifuge at 500 *x g* for 5 minutes at room temperature. Following centrifugation, the supernatant was removed, and the cell pellet was resuspended in fresh 10% RPMI at the required density before seeding the cells into culture plates.

2.2.3 MTS Assay

The CellTiter 96® AQ_{ueous}One Solution Cell Proliferation Assay (Promega, UK) is a colorimetric method used to determine the number of viable cells in cytotoxicity and proliferation assays. The solution contains the novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), which is reduced by metabolically active cells to form a soluble formazan product. This product is directly proportional to the number of viable cells in culture (Promega Corporation, 2012).

A suspension of Jurkat cells was prepared as per Section 2.2.2, and aliquoted into a 96 well flat-bottomed plate (100 µL; 2.0x10^5 cells/well). Five concentrations of each lignan compound were prepared as described in Section 2.2.1, and 100 µL of each concentration was added to the appropriate cell suspensions at final concentrations

of either 200 µM, 100 µM, 50 µM, 25 µM or 12.5 µM per well. Untreated and non-viable controls were also prepared by treating the cell suspension with 100 µL of 10% RPMI and absolute ethanol, respectively. All treatments were performed in triplicate, except the untreated controls which had six replicates. The plate containing the cells was incubated at 37 °C in a 5% CO_2 humidified incubator for either 24 hours, or 48 hours.

Following incubation, the contents of each well were placed in 0.5 mL microcentrifuge tubes and centrifuged at 500 *x g* for 5 minutes at room temperature. The supernatant was removed, and the pellet was displaced with 100 µL of fresh 10% RPMI. The mixtures were replaced into a 96 well flat-bottomed plate, with 20 µL of CellTiter 96® AQ_{ueous}One Solution Cell Proliferation Assay added to each well. The plate was protected from the light with foil and incubated at 37 °C in a 5% CO_2 humidified environment for 2.5 hours. The plate was analysed using a Synergy HT plate reader (BioTek, UK) which measured the absorbance of the solutions at 450 nm.

2.2.4 Annexin V/Propidium Iodide Assay

The Annexin V/PI assay is a bivariate flow cytometric assay which is used to distinguish the mode of cell death within a sample. Annexin V, a Ca^{2+}-dependent protein that has high affinity for the membrane protein PS, is conjugated with a FITC fluorochrome, which can be used to analyse apoptosis with flow cytometry. This is performed in conjunction with propidium iodide (PI), a dye that intercalates with DNA. During necrosis and the later stages of apoptosis, the plasma membrane becomes permeable to PI, therefore the nucleus of these cells will fluoresce red (Vermes et al., 2000). Photons are emitted following excitation by the laser and are detected by photomultiplying tubes, and by measuring the presence or absence of Annexin V and PI, can be categorized as viable, early apoptotic, late apoptotic or necrotic (Table 2.2.4.1).

A suspension of Jurkat cells was prepared as described in Section 2.2.2, and aliquoted into a 96 well flat-bottomed plate (100 µL; $1.0x10^5$ cells/well). Three concentrations of each lignan compound were prepared as per Section 2.2.1, and 100 µL of each concentration was added to the appropriate cell suspensions at final

concentrations of either 200 μM, 100 μM or 50 μM per well. Each concentration of lignan compound was incubated with the Jurkat cells in triplicate. Untreated controls were also performed using 100 μL of 10% RPMI media, in place of the lignan compound solutions. The plate containing the cells was incubated at 37 °C in a 5% CO_2 humidified incubator for 24 hours.

The cells were removed from the wells and placed in 1.5 mL microcentrifuge tubes and were spun in a centrifuge at 500 *x g* for 5 minutes at room temperature. The supernatant was removed, and the pellet was resuspended in 300 μL of Dulbecco's Phosphate Buffered Saline (DPBS; Lonza, UK) and centrifuged again at 500 *x g* for 5 minutes at room temperature. The supernatant was removed, and the pellet was resuspended in 100 μL of 1X Annexin V Binding Buffer (BD Biosciences, UK), then transferred to a capped 5mL polystyrene round bottomed tube (Corning, UK). A 15 μL aliquot from master mix containing 2:1 Propidium Iodide to Annexin V-FITC (both BD Biosciences, UK) was added to each tube, mixed, and incubated in the dark for 15 minutes at room temperature. An additional 400 μL of 1X Annexin V Binding Buffer was added to the tubes, and the contents were immediately analysed using a BD FACSVerse™ flow cytometer and FACSuite™ analysis software (both BD Biosciences, UK).

Initially, the Jurkat cell population was identified and gated using forward scatter (FSC) and side scatter (SSC) of a sample with no fluorochromes present (unstained). This gate was applied to each sample that was tested (Figure 2.2.4.1).

Once identified, the population was gated in order to discard the debris, and the voltages of the photomultiplier tubes were adjusted to remove background fluorescence of FITC and PI. Compensation controls containing single stains of either Annexin V-FITC or PI were then analysed to calculate and adjust for spectral overlap. The experimental samples were then analysed and were grouped according to the results as shown in Table 2.2.4.1.

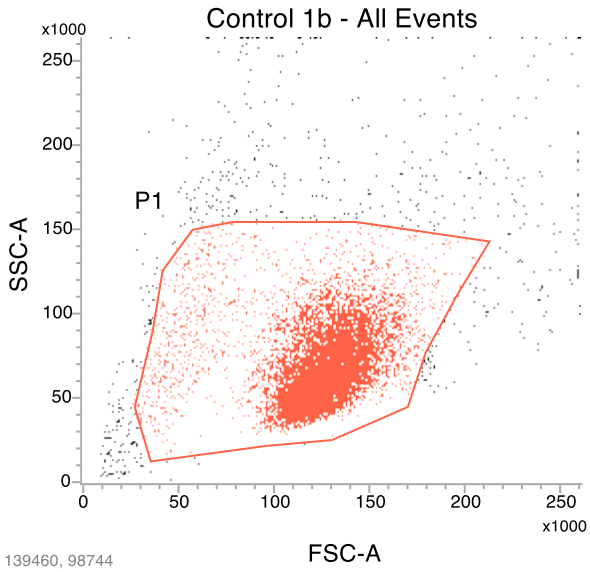


Figure 2.2.4.1: ***Dotplot of events following flow cytometric acquisition of an untreated control sample.*** *Gate "P1" selects the Jurkat cell population based on Side Scatter (SSC) and Forward Scatter (FSC), which was applied to each sample analysed.*

Table 2.2.4.1: ***Viability of cells according to measured fluorescence.***

	Annexin V-FITC	Propidium Iodide
Viable	-	-
Early Apoptotic	+	-
Late Apoptotic	+	+
Necrotic	-	+

2.2.5 Cell Cycle Analysis

The stages of cell cycle progression can be categorised by measuring DNA content within cells using the DNA-intercalating agent PI, which can be quantified using a flow cytometer. The cells can be categorised as either G_1, S, or G_2/M phase with a DNA content of 2n, >2n and 4n, respectively (Figure 2.2.5.1) (Nicoletti et al., 1991; Riccardi and Nicoletti, 2006).

A suspension of Jurkat cells as prepared as described in Section 2.2.2, and aliquoted into a 48 well flat-bottomed plate (200 µL; $1.2x10^6$ cells/well). The lignan compounds were prepared as per Section 2.2.1, and 200 µL of each concentration was added to the appropriate cell suspensions at a final concentration of 100 µM per well. Untreated controls were also performed, incubating the Jurkat cells with 200 µL of

10% RPMI instead of lignan compound. Additionally, positive controls were performed, using 0.5 µM camptothecin to induce cell cycle arrest.

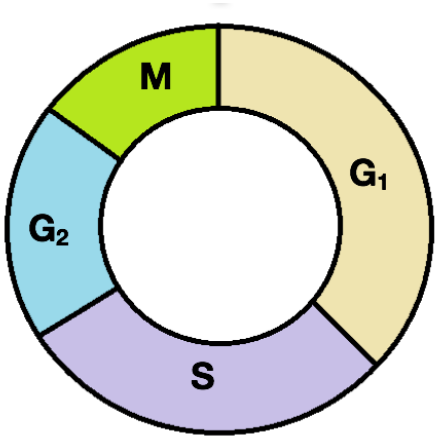


Figure 2.2.5.1: ***Diagram indicating cell cycle progression through interphase and the mitotic phase – adapted from Williams and Stoeber, 2011.***

All treatments and controls were performed in triplicate. The plate containing the cells was then incubated at 37 °C in a 5% CO_2 humidified incubator for 4 hours. Shorter incubations were chosen for cell cycle analysis to observe the initial stages of lignan compound exposure to the Jurkat cells.

Following incubation, the cells were removed from the plate and replaced in 1.5 mL microcentrifuge tubes. The samples were spun in a centrifuge at 500 *x g* for 5 minutes at room temperature, and once completed the supernatant from the samples were discarded, leaving the pellet. The pellet was vortexed while simultaneously adding 500 µL 70% ethanol dropwise, fixing the cells and minimising clumping. The samples were incubated at 4 °C for 30 minutes, and then centrifuged at 1000 *x g* for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 500 µL DPBS. The samples were spun again at 1000 *x g* for 5 minutes, and the supernatant was removed a final time. The pellet was resuspended in 50µL RNase A (100 µg/mL stock; Roche, UK) and 200 µL PI (50 µg/mL stock; Sigma, UK). The samples were then analysed using a BD FACScalibur™ flow cytometer with BD CellQuest™ Pro analysis software (both BD Biosciences, UK).

The Jurkat cell population was detected by measuring the FSC/SSC of the sample, and gating the cells, which excluded the majority of debris from further analysis.

Within this population, another gate was constructed using FL2-A/FL2-W that included cells twice the area which characterised mitosis but excluded cells twice the width which would indicate cell clumping (Figure 2.2.5.1).

The data produced following analysis of the samples was then processed using ModFit LT™ v5.0.9 (Verity House Software, USA), which modelled the raw data and categorized the number of cells in G_1, S, or G_2/M-phase.

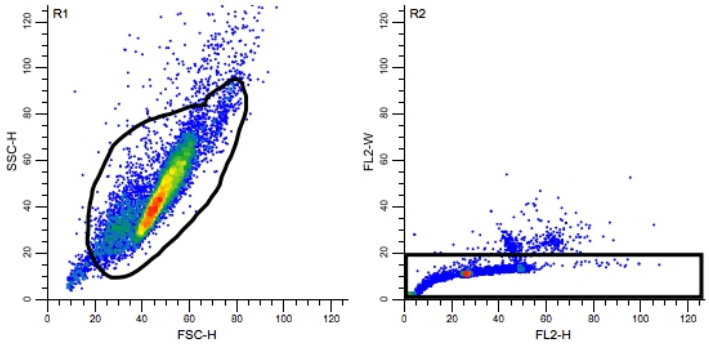


Figure 2.2.5.1: ***Dotplots of events following flow cytometric acquisition of an untreated control sample.*** *Region "R1" selects the Jurkat cell population based on Side Scatter (SSC) and Forward Scatter (FSC), while region "R2" excludes cell doublets from analysis using FL2-W and FL2-A.*

2.2.6 Western Blotting

Western Blotting is a technique which can determine the expression of proteins in extracts or cell lysates, by detecting the fluorescence of antibodies which bind to the epitope of the protein of interest.

2.2.6.1 Protein Extraction

A suspension of Jurkat cells was prepared as per Section 2.2.2, and aliquoted into a 12 well flat-bottomed plate (600 µL; $1.5x10^6$ cells/well). The lignan compounds were prepared as per Section 2.2.1, and 600 µL of each concentration was added to the appropriate cell suspensions at a final concentration of 100 µM per well. Additionally, Jurkat cells were incubated with 600 µL of 10% RPMI in place of lignan compound to act as an untreated control. The plate containing the cells was then incubated at 37 °C in a 5% CO_2 humidified incubator for 4 hours.

The contents of the wells were replaced in 1.5 mL microcentrifuge tubes, and the samples were spun in a centrifuge at 500 *x g* for 5 minutes at room temperature. The

supernatant was discarded, and the pellet was resuspended in 120 µL radioimmunoprecipitation assay buffer (RIPA buffer; made in house) containing freshly added 1:1000 Protease Inhibitor Cocktail and 1:1000 Phosphatase Inhibitor Cocktail (both Sigma, UK). The samples were resuspended using the micropipette, and incubated on ice for 15 minutes. The samples were intermittently sonicated for 10 seconds at room temperature. The samples were spun in a centrifuge at 13,500 *x g* at 4 °C for 20 minutes. The lysate was transferred from the samples and replaced in chilled 1.5 mL microcentrifuge tubes, and the pellet was discarded.

2.2.6.2 Protein Assay and Quantification

The concentration of protein in the lysate of each sample was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK). Known standard concentrations of bovine serum albumin (BSA; included in the assay kit) were diluted in RIPA extraction buffer to 125-2000 µg/mL, and a blank reference sample containing only extraction buffer. A 10 µL volume of each standard and sample lysate was placed in a 96 well flat-bottomed plate and mixed with 200 µL working reagent (made by combining Reagent A and Reagent B from the assay kit at 50:1 ratio). The plate was incubated at 37 °C in a 5% CO_2 humidified incubator for 30 minutes.

Following incubation, the plate was removed and allowed to cool to room temperature. The plate was analysed using a Bio-Tek Synergy HT Plate Reader, and the absorbance of each sample of unknown protein concentration was compared to the absorbance of the known standards at 562 nm. The protein concentration of the samples was determined and diluted with extraction buffer appropriately to ensure uniform protein concentration across the samples. The samples were then aliquoted and stored at -20 °C until required.

2.2.6.3 Gel Electrophoresis and Transfer

Resolving gels were prepared (as per Section 2.1.5) and were incubated for 45 minutes at room temperature, covered with a layer of distilled water (dH_2O) to prevent evaporation. Once set, the layer of water was removed, and the stacking gel (as per Section 2.1.5) was poured on top of the resolving gel, with 10-well combs placed in the stacking gel. The gels were incubated at room temperature for 20 minutes.

The required aliquots of cell lysate were defrosted on ice, and 1 part NuPAGE™ LDS 4X Sample Buffer (Thermo Fisher Scientific, UK) was added to 3 parts cell lysate. The samples were heated at 75 °C for 10 minutes, and then returned to the ice and allowed to cool.

The plates were transferred to a gel tank, and the core was filled with 1X running buffer (as per Section 2.1.5) to check for leaking. The tank was filled to the required volume with additional 1X running buffer, and the combs were removed from the gels. The outer wells on each gel were filled with 5 µL sample buffer, and 5 µL pre-stained molecular weight marker (Bio-Rad, UK) was added. A 20 µL volume of each sample was added to the gels and run at 115 V for 1 hour and 15 minutes at room temperature.

The gels were transferred to nitrocellulose membranes using the TransBlot® Turbo™ Transfer System (Bio-Rad, UK) at 25 V (2.5 A) for 7 minutes.

2.2.6.4 Blocking, Antibody Incubation and Detection

The membranes were blocked for 1 hour using 5% BSA dissolved in Tris Buffered Saline (TBS; made in-house) with 0.1 % Tween®-20 (Sigma). The 5% BSA was removed, and the membranes were each placed in 50 mL centrifuge tubes and incubated with 1:1000 required primary antibody (refer to Section 2.1.5), diluted in 2.5 % BSA in TBS-Tween. The tubes were continuously rolled at either 4 °C overnight or at room temperature for 4 hours. The membranes were washed four times in TBS-Tween and incubated with 1:5000 required secondary HRP-linked antibody (refer to Section 2.1.5) for 1 hour at room temperature. β-Actin was used as the loading control for all detections. The membranes were washed four times in TBS-Tween and incubated with 1:1 Pierce enhanced chemiluminescence kit (ECL; Thermo Fisher Scientific, UK) for 2 min. Excess ECL was drained, and each membrane was examined for chemiluminescence using a Bio-Rad Chemidoc Touch Imager.

2.2.6.5 Densitometry

Western Blot images were analysed using ImageLab software (Bio-Rad, UK). The present bands were identified, and the total band volume was calculated.

The loading control across each lane was normalised and compared with the volume of the bands produced by the protein of interest. Each ratio was compared with the ratio of the untreated control sample, thus calculating the relative expression of protein.

2.3 Statistical Analysis

Statistical analysis was performed on all data using GraphPad PRISM® version 5.0a for Mac. The one-way analysis of variance (ANOVA) was used to compare more than two groups of data, and Dunnet's post-hoc test was chosen to compare each test group with the control group. Where significant differences were found, * represents a p value <0.05, ** represents a p value <0.01, and *** represents a p value <0.001.

3.0 Results

3.1 Effect of novel lignan compound treatment on cellular metabolism of Jurkat cells

The metabolic activity of the Jurkat cells after treatment with each lignan compound were compared to an untreated control to determine significance (Section 2.2.3). The mean absorbances of the treatments were normalised to percentages, using the non-viable controls and the untreated controls as 0% and 100% standards, respectively.

The metabolic activity of the Jurkat cells when treated with 200 μM of each lignan compound following incubation for 24 hours is shown (Figure 3.1.1). Lignan compounds SD007, SD010, SD012 and SD013 demonstrated significantly lower metabolic activity following treatment ($P<0.001$) compared to the control. Additionally, the metabolic activity of SD008, SD009 and SD014 were shown to be significantly decreased ($P<0.01$), as was SD002, SD006 and SD015 ($P<0.05$). The metabolic activity of the Jurkat cells following a 24 hours incubation with 100 μM of lignan compounds SD007 and SD013 were significantly decreased ($P<0.001$ and $P<0.01$, respectively) from the control (Figure 3.1.2). These were the only lignan compounds that significantly affected metabolic activity following treatment at 100 μM concentration. Treating Jurkat cells with some lignan compounds at 12.5μM significantly increased the metabolic activity (Figure 3.1.2 C and D both $P<0.05$).

To summarise, treating the Jurkat cells for 24 hours with 200 μM of lignan compounds SD007-10 and SD012-15 resulted in a significant reduction of metabolic activity. Treating the Jurkat cells for 24 hours with 100 μM of lignan compounds SD007 and SD013 also significantly decreased the metabolic activity of the Jurkat cells.

Figure 3.1.2A-D shows lignan compounds SD007, SD008 SD012 and SD013 to give a general overview of the varied results after treatment with different concentrations of the lignan compounds. Refer to the appendices for the graphs of all lignan compounds (Table 7.2), in addition to the absolute metabolic activity values (Table 7.1).

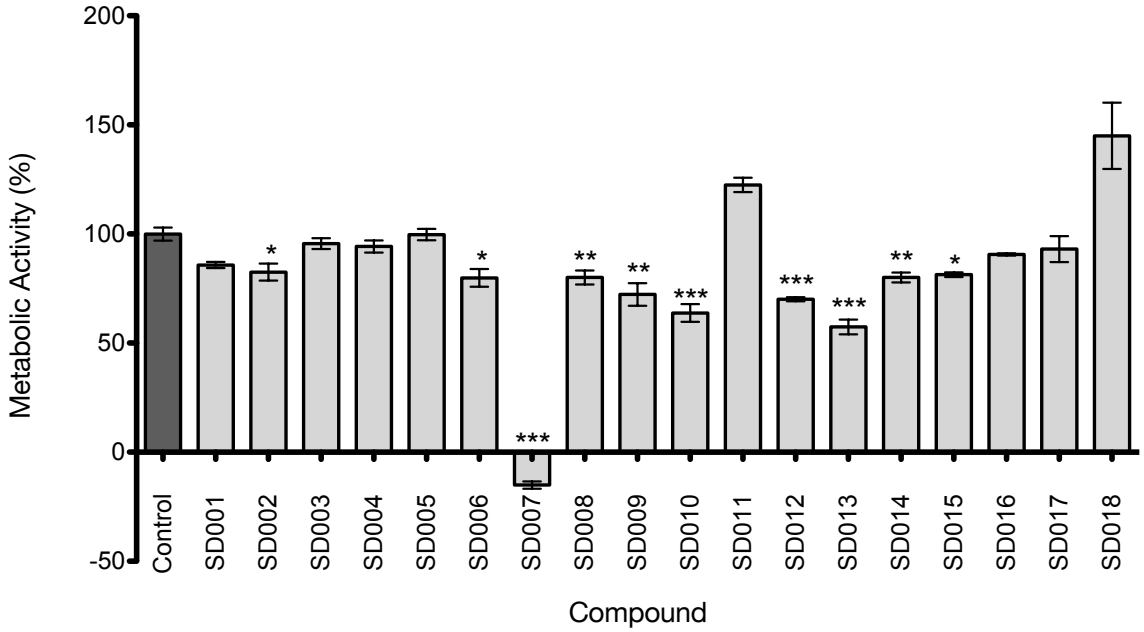


Figure 3.1.1: ***Metabolic activity of Jurkat cells following treatment for 24 hours with 200 μM of each lignan compound.*** *Data is represented as normalised mean, ± SEM. Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*

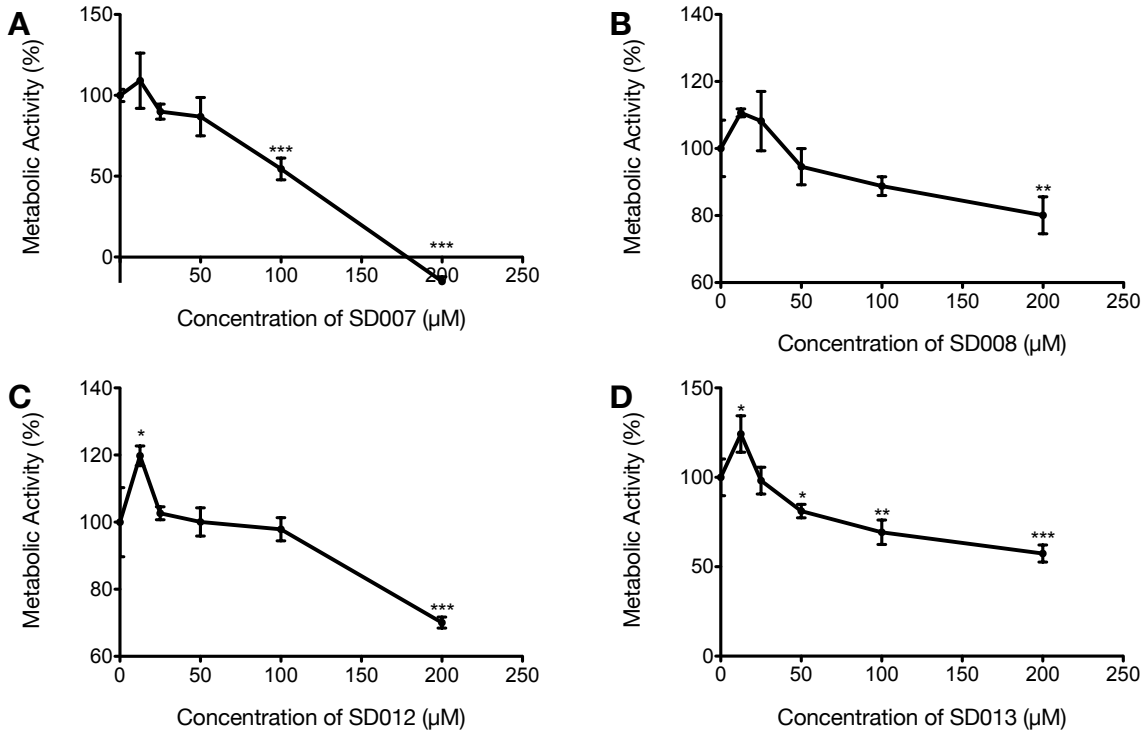


Figure 3.1.2: ***Metabolic activity of Jurkat cells incubated for 24 hours with 12.5-200 μM of lignan compounds SD007 (A), SD008 (B), SD012 (C) and SD013 (D).*** *Data is represented as normalised mean, ± SEM. Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*

The data from the 24-hour time point experiments indicated which lignan compounds inhibited the growth of the Jurkat cells. To provide further information on the effect of the lignan compounds on the metabolic activity of Jurkat cells, the cells were treated for 48 hours. Figure 3.1.3 and 3.1.4 give a general overview of the results from this time point, refer to the appendices (Tables 7.4, 7.5 and 7.6) for graphs and absolute data for each lignan compound. The metabolic activity of the Jurkat cells following treatment with 200 μM of lignan compounds SD002 (Figure 3.1.4 A), SD003-8, SD010-12, SD014, and SD016-17 was significantly decreased (Table 7.4) when compared to the control. Significantly lower metabolic activity was also observed when the Jurkat cells were treated with SD001 ($P<0.01$), SD013 and SD018 ($P<0.05$). Treatment using lignan compound SD015 demonstrated no significant reduction in metabolic activity (Figure 3.1.4 B), and the supply of lignan compound SD009 had ran out before the experiment took place.

The results show that at 200 μM, there was a significant reduction in metabolic activity when treating the Jurkat cells with lignan compounds SD002, SD006-10 and SD012-15 when incubating for 24 h. When the Jurkat cells were treated with 100 μM of lignan compounds SD007 and SD013, there continued to be a significant decrease in metabolic activity (refer to appendices, Tables 7.4 & 7.5). There was also an increase in metabolic activity of the Jurkat cells when incubating with lower concentrations with some lignan compounds, occasionally significantly (Table 7.5).

Treating the Jurkat cells for 48 hours resulted in a significant decrease in metabolic activity when incubating with 200 μM of each lignan compound, with the exception of SD015.

The results from the MTS assays were preliminary data that highlighted which particular lignan compounds had anti-cancer potential and were good candidates for further research later in the project.

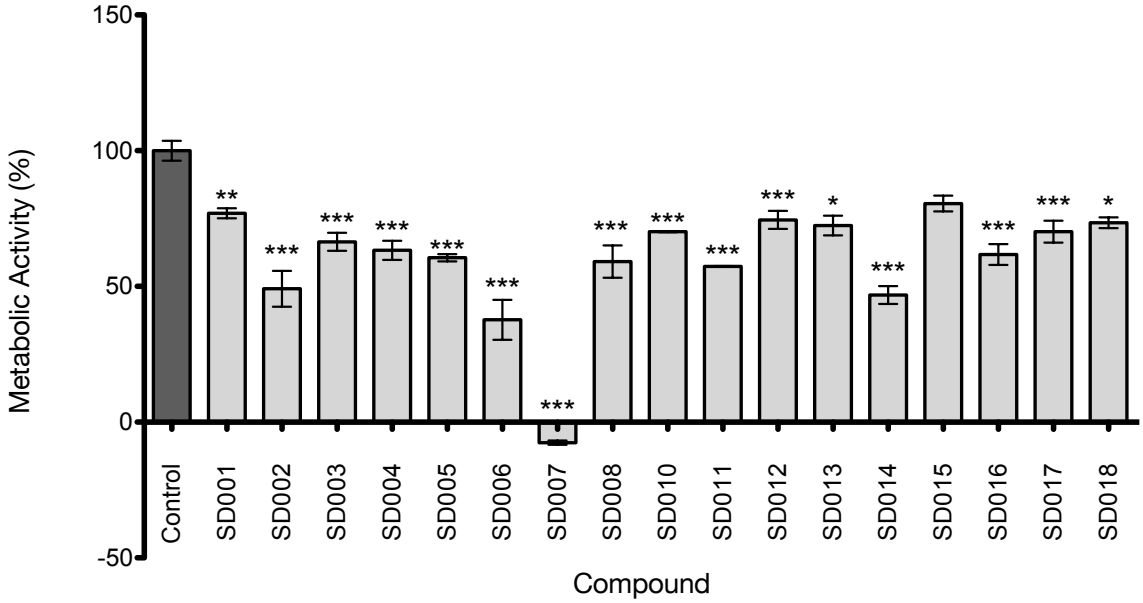


Figure 3.1.3: ***Metabolic activity of Jurkat cells following treatment for 48 hours with 200 μM of each lignan compound.*** *Data is represented as normalised mean, ± SEM. Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*

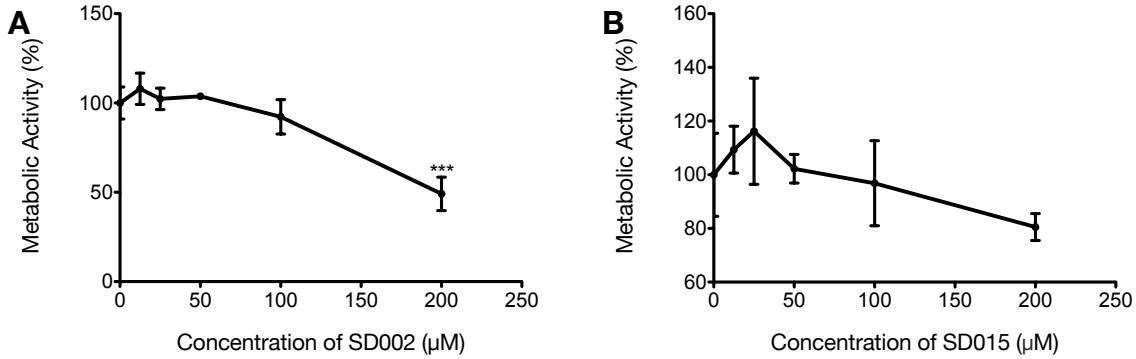


Figure 3.1.4: ***Metabolic activity of Jurkat cells incubated for 48 hours with 12.5-200 μM of lignan compounds SD002 (A) and SD015 (B).*** *Data is represented as normalised mean, ± SEM. Statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test. *** represents a p value < 0.001.*

3.2 Effect of lignan compounds on apoptosis and necrosis of Jurkat cells following treatment

Results from the MTS assays showed that several lignan compounds had a significant effect on cell metabolism, but those that did not could have caused early cellular events that do not extrapolate to a change in metabolic activity.

Following treatment by various concentrations of the lignan compounds for 24 hours, the Jurkat cells were prepared for analysis by flow cytometry (Section 2.2.4). The Jurkat cell population was selected and gated into four viability stages; viable, early apoptotic, late apoptotic and necrotic. Figure 3.2.1 is an example of the flow cytometry results following the Annexin V/PI assay and Table 3.2.1 summarises the results of the assay following Jurkat cell treatment with the bank of lignan compounds.

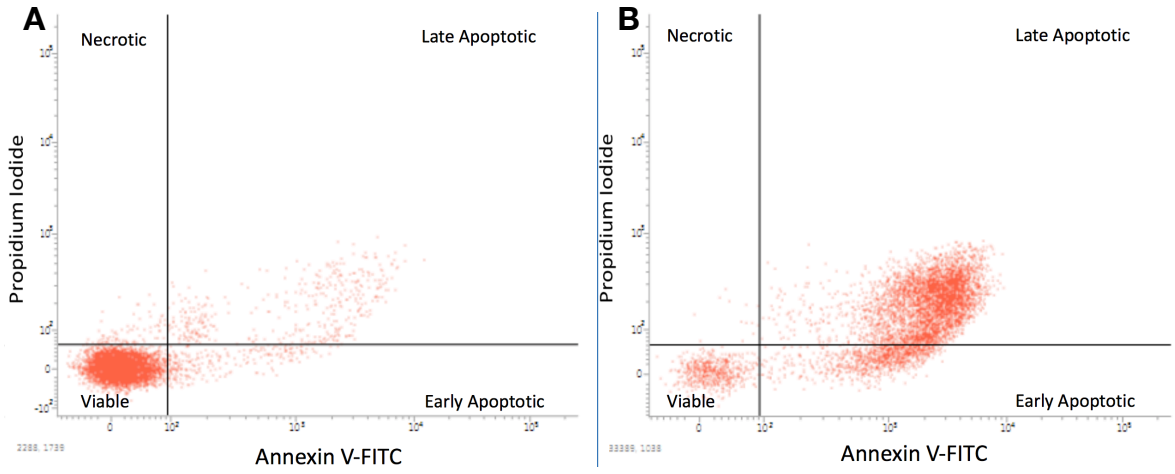


Figure 3.2.1: *Dotplots generated by FACSuite showing viable, early apoptotic, late apoptotic and necrotic cells of untreated control (A) and treated with 200 µM SD007 for 24 hours (B).*

Table 3.2.1: Summary of data following treatment of Jurkat cells with 50-*200 μM of each lignan compound for 24 hours*. The percentage of Jurkat cells in each stage of viability are shown, with significance of the result compared to the untreated control indicated if applicable. Refer to the Appendices (Table 7.7) for further information. *Data is represented as mean, statistical analysis was performed using the one-way ANOVA with Dunnett's post-hoc test. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*

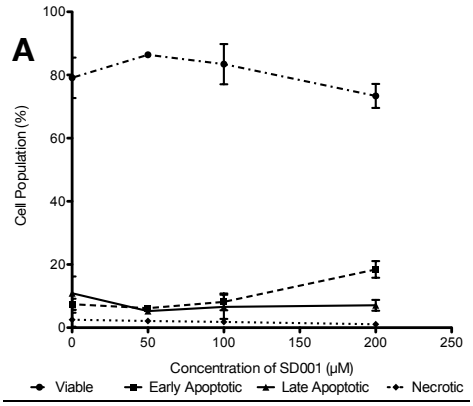
Lignan	Viable			Early Apoptotic			Late Apoptotic			Necrotic		
	50 μM	100 μM	200 μM	50 μM	100 μM	200 μM	50 μM	100 μM	200 μM	50μM	100 μM	200 μM
SD001	86.4%	83.4%	73.4%	6.2%	8.2%	18.4%***	5.3%	6.6%	7.1%	2.1%	1.8%	1.1%
SD002	84.8%	85.6%	68.3%*	5.9%	6.8%	22.5%***	6.1%	5.8%	8.5%	3.2%	1.8%	0.8%
SD003	83.9%	80.9%	74.1%	9.5%	11.6%**	17.7%***	5.1%	6.3%	6.7%	1.5%	1.3%	1.5%
SD004	76%	78.2%	68.1%	9.8%	9.5%*	17%***	13.2%	11.5%	13.8%	1.1%	0.7%	1.1%
SD005	82.4%	83.6%	64.3%	6.7%	6.5%	17.8%***	5.4%	4.5%	11.2%	5.7%	5.1%	6.7%
SD006	78.8%	79.2%	72.5%	8.2%	8.1%	12.7%***	11.2%	10.9%	13.2%	1.9%	1.8%	1.6%
SD007	75.9%	72.4%**	5.8%***	10.6%*	10%	27%***	10.6%	14%	62.8%***	3.2%	3.6%	1.2%
SD008	73.9%	73.9%	49.8%***	12%**	11.8%**	28.5%***	12.5%	12.9%	20.6%***	1.7%	1.4%***	1.1%
SD009	72.8%	72.6%	46.5%***	13.2%**	13.2%**	35.2%***	12.5%	12.8%	17.7%**	1.5%	1.4%	0.7%
SD010	70.7%	62.1%**	46.3%***	14.4%*	17%**	24.8%***	13.5%	19.6%*	27.9%***	1.5%	1.3%	1%*
SD011	75.8%	76.2%	76.3%	10.7%**	11.7%***	12.2%***	10.5%	9.7%	10.6%	3%	2.5%	0.9%
SD012	72.6%***	65.7%***	35.9%***	7.2%*	8.1%***	13.7%***	19%***	24.1%***	49%***	1.3%	2.1%*	1.4%
SD013	74.9%***	41.2%***	34.4%***	11.7%***	15.6%***	16.4%***	12.8**	40.7***	44.2***	0.6	2.5*	5%***
SD014	75.9%***	72.3%***	55%***	12.4%***	11.5%***	7.3%**	10.5%	14.6%**	34.6%***	1.2%	1.5%	3%**
SD015	79.8%*	76.4%***	76%***	11.3%**	12.2%**	8.7%***	8.4%	10.6%	14.6%***	0.5%*	0.8%	0.8%
SD016	74.9%***	69.8%***	67.2%***	17.2%***	18.7%***	13.5%***	7%	10%	17.9%***	0.9%	1.5%	1.4%
SD017	87.5%	83.7%	84.5%	5.4%	7.9%	8.5%	6%	7.3%	6.3%	1.1%	1.1%***	0.7%***
SD018	67.8%***	47.2%***	37.4%***	23.3%***	18.2%***	19.7%***	8.5%	33%***	40.5%***	0.4%	1.7%	2.4%**

There was a significant decrease in the percentage of viable cells compared to the control following treatment of the Jurkat cells with 200 μM of lignan compounds SD002, SD007-10, SD012-16 and SD018 (refer to appendices Table 7.7 for absolute values). Treating the Jurkat cells with 100 μM of lignan compounds SD007, SD010, SD012-16 and SD018 showed a significantly reduced percentage of viable Jurkat cells. Furthermore, treatment with 50 μM of lignan compounds SD012-16 and SD018 continued to significantly reduce the percentage of viable Jurkat cells (Table 3.2.1; Figures 3.2.3-3.2.6).

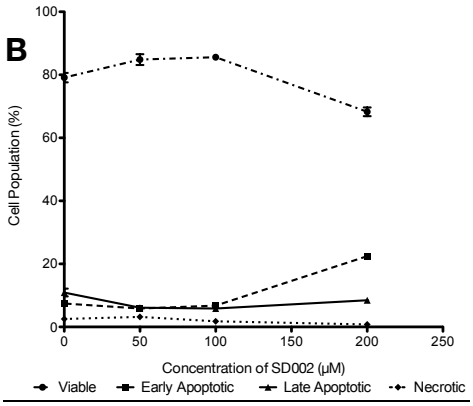
The percentage of early apoptotic cells was significantly higher than the untreated control when the Jurkat cells were treated with 200 μM of lignan compounds SD001-16 and SD018. Following treatment with 100 μM of lignan compounds SD003, SD004, SD008-16 and SD018, the percentage of early apoptotic Jurkat cells was also significantly increased. There was also a significantly increased percentage of early apoptotic Jurkat cells when treated with 50 μM of lignan compounds SD007-16 (Table 3.2.1; Figures 3.2.2-3.2.6).

The percentage of Jurkat cells in late apoptosis following treatment with 200 μM of lignan compounds SD007-10, SD012-16 and SD018 was significantly increased compared to the untreated control. Following treatment with 100 μM of lignan compounds SD010, SD012-14 and SD018, there was a significant increase in late apoptotic Jurkat cells. Treatment with 50 μM of lignan compounds SD012 and SD013 showed a significantly increased number of late apoptotic Jurkat cells (Table 3.2.1; Figures 3.2.2-3.2.6).

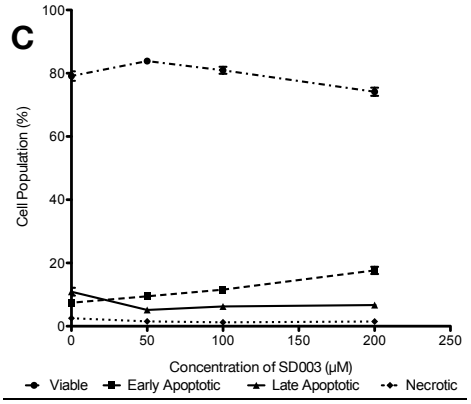
The percentage of necrotic Jurkat cells was significantly decreased compared to the untreated control following treatment with 200 μM of lignan compounds SD010 and SD017, 100 μM of lignan compound SD017 and 50 μM of lignan compound SD015. The percentage of necrotic Jurkat cells significantly increased following treatment with 200 μM lignan compounds SD013-14 and SD018. A significant increase in the percentage of necrotic Jurkat cells was also observed following treatment with 100 μM of lignan compounds and SD012 and SD013 (Table 3.2.1; Figures 3.2.3-3.2.4).



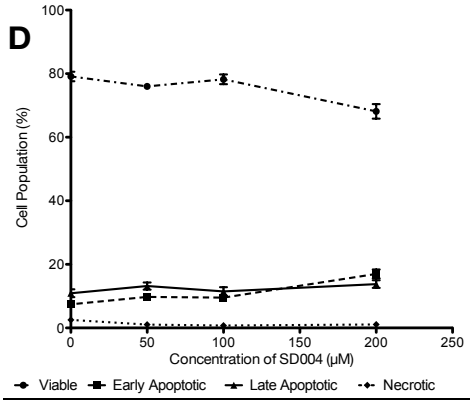
	50μM	100μM	200μM
Viable	n.s.	n.s.	n.s.
Early Apoptotic	n.s.	n.s.	***
Late Apoptotic	n.s.	n.s.	n.s.
Necrotic	n.s.	n.s.	n.s.



	50μM	100μM	200μM
Viable	n.s.	n.s.	*
Early Apoptotic	n.s.	n.s.	***
Late Apoptotic	n.s.	n.s.	n.s.
Necrotic	n.s.	n.s.	n.s.

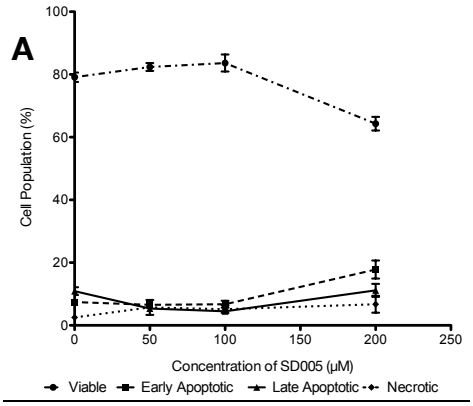


	50μM	100μM	200μM
Viable	n.s.	n.s.	n.s.
Early Apoptotic	n.s.	**	***
Late Apoptotic	n.s.	n.s.	n.s.
Necrotic	n.s.	n.s.	n.s.

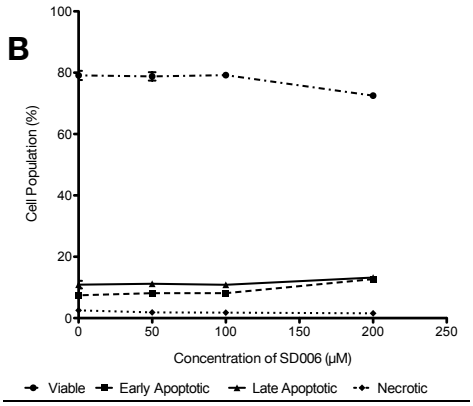


	50μM	100μM	200μM
Viable	n.s.	n.s.	n.s.
Early Apoptotic	n.s.	*	***
Late Apoptotic	n.s.	n.s.	n.s.
Necrotic	n.s.	n.s.	n.s.

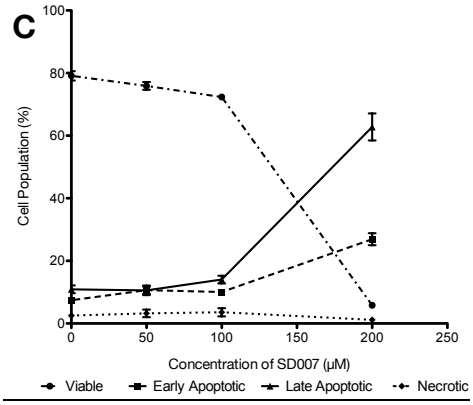
Figure 3.2.2: ***Stages of Jurkat cell viability following treatment for 24 hours with 50-200 μM of lignan compounds SD001 (A), SD002 (B), SD003 (C) and SD004 (D).*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*



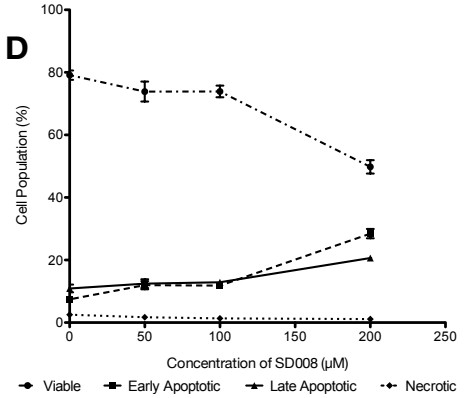
	50μM	100μM	200μM
Viable	n.s.	n.s.	n.s.
Early Apoptotic	n.s.	n.s.	***
Late Apoptotic	n.s.	n.s.	n.s.
Necrotic	n.s.	n.s.	n.s.



	50μM	100μM	200μM
Viable	n.s.	n.s.	n.s.
Early Apoptotic	n.s.	n.s.	***
Late Apoptotic	n.s.	n.s.	n.s.
Necrotic	n.s.	n.s.	n.s.

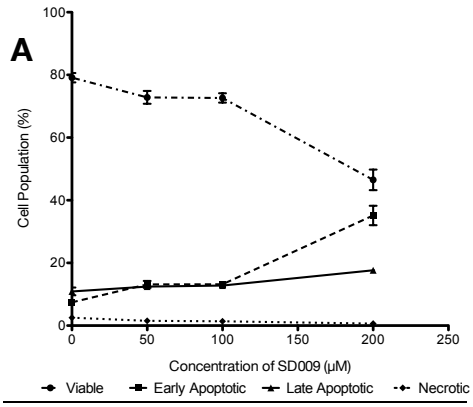


	50μM	100μM	200μM
Viable	n.s.	**	***
Early Apoptotic	*	n.s.	***
Late Apoptotic	n.s.	n.s.	***
Necrotic	n.s.	n.s.	n.s.

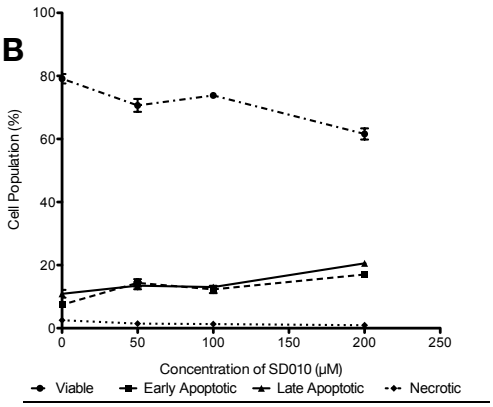


	50μM	100μM	200μM
Viable	n.s.	n.s.	***
Early Apoptotic	**	**	***
Late Apoptotic	n.s.	n.s.	***
Necrotic	n.s.	n.s.	n.s.

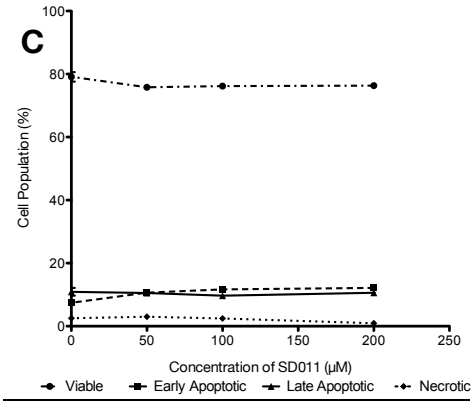
Figure 3.2.3: ***Stages of Jurkat cell viability following treatment for 24 hours with 50-200 μM of lignan compounds SD005 (A), SD006 (B), SD007 (C) and SD008 (D).*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*



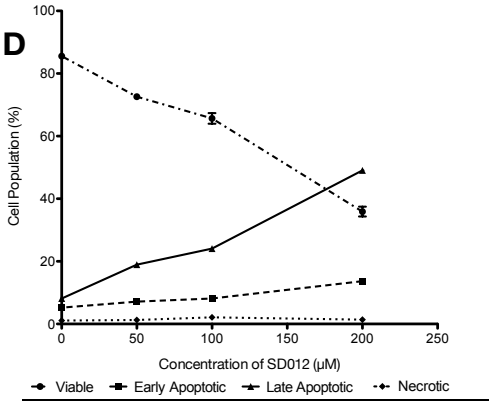
	50μM	100μM	200μM
Viable	n.s.	n.s.	***
Early Apoptotic	**	**	***
Late Apoptotic	n.s.	n.s.	**
Necrotic	n.s.	n.s.	n.s.



	50μM	100μM	200μM
Viable	n.s.	**	***
Early Apoptotic	*	**	***
Late Apoptotic	n.s.	*	***
Necrotic	n.s.	n.s.	*

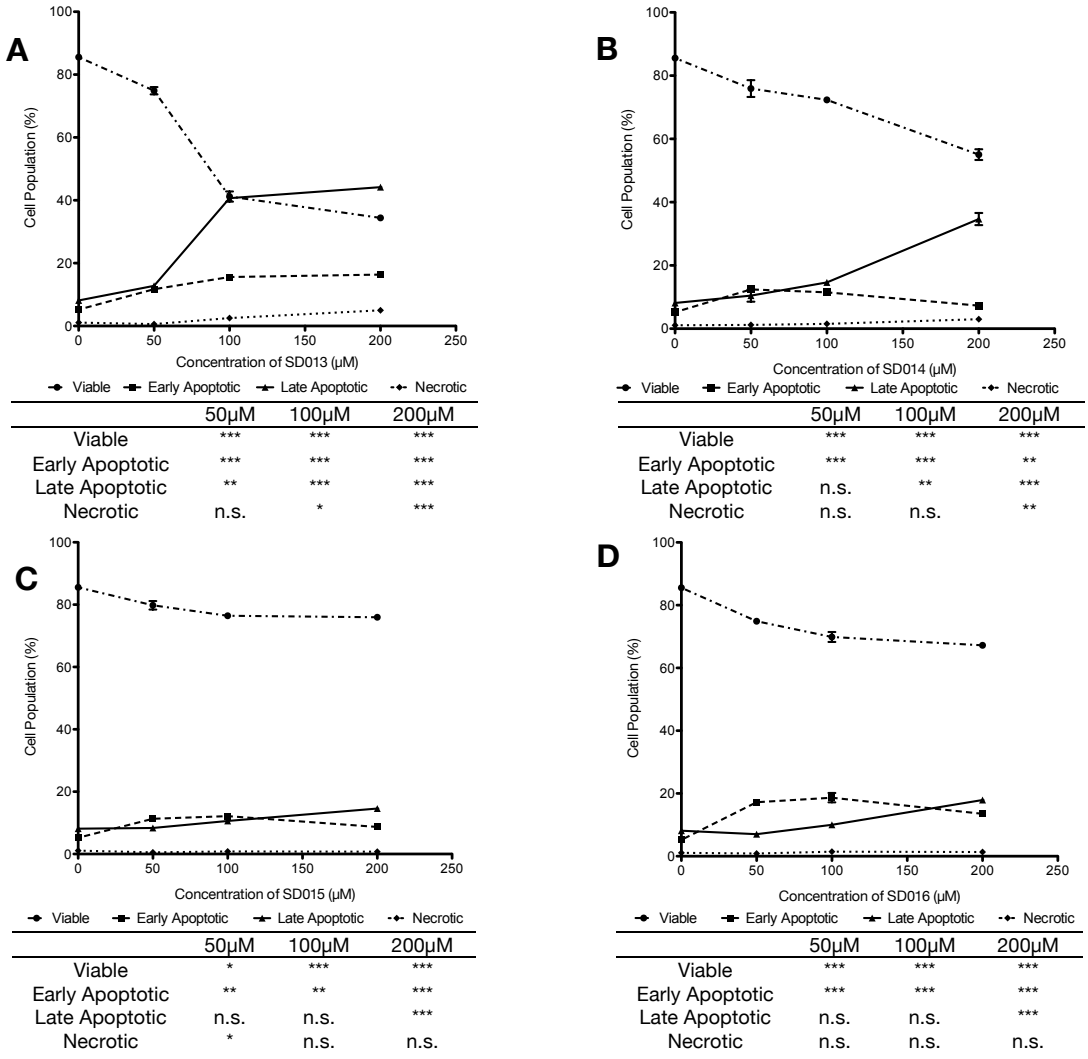


	50μM	100μM	200μM
Viable	n.s.	n.s.	n.s.
Early Apoptotic	**	***	***
Late Apoptotic	n.s.	n.s.	n.s.
Necrotic	n.s.	n.s.	n.s.



	50μM	100μM	200μM
Viable	***	***	***
Early Apoptotic	*	***	***
Late Apoptotic	***	***	***
Necrotic	n.s.	*	n.s.

Figure 3.2.4: ***Stages of Jurkat cell viability following treatment for 24 hours with 50-200 μM of lignan compounds SD009 (A), SD010 (B), SD011 (C) and SD012 (D).*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*



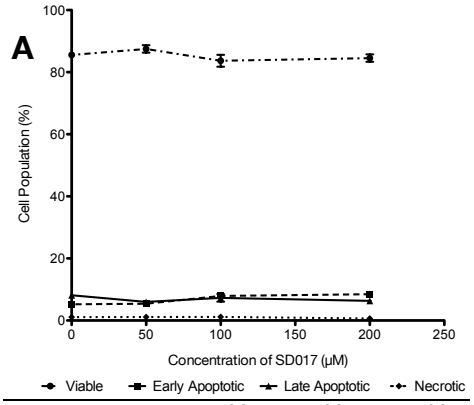
A	50μM	100μM	200μM
Viable	***	***	***
Early Apoptotic	***	***	***
Late Apoptotic	**	***	***
Necrotic	n.s.	*	***

B	50μM	100μM	200μM
Viable	***	***	***
Early Apoptotic	***	***	**
Late Apoptotic	n.s.	**	***
Necrotic	n.s.	n.s.	**

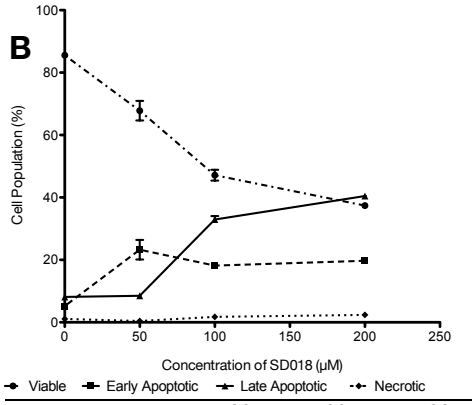
C	50μM	100μM	200μM
Viable	*	***	***
Early Apoptotic	**	**	***
Late Apoptotic	n.s.	n.s.	***
Necrotic	*	n.s.	n.s.

D	50μM	100μM	200μM
Viable	***	***	***
Early Apoptotic	***	***	***
Late Apoptotic	n.s.	n.s.	***
Necrotic	n.s.	n.s.	n.s.

Figure 3.2.5: ***Stages of Jurkat cell viability following treatment for 24 hours with 50-200 μM of lignan compounds SD013 (A), SD014 (B), SD015 (C) and SD016 (D).*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*



	50μM	100μM	200μM
Viable	n.s.	n.s.	n.s.
Early Apoptotic	n.s.	n.s.	n.s.
Late Apoptotic	n.s.	n.s.	n.s.
Necrotic	n.s.	***	***



	50μM	100μM	200μM
Viable	***	***	***
Early Apoptotic	***	***	***
Late Apoptotic	n.s.	***	***
Necrotic	n.s.	n.s.	**

Figure 3.2.6: ***Stages of Jurkat cell viability following treatment for 24 hours with 50-200 μM of lignan compounds SD017 (A) and SD018 (B).*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*

3.3 Effect of novel lignan compound treatment on the cell cycle distribution of Jurkat cells following treatment

The results of the apoptosis and necrosis assay indicated that a number of lignan compounds induced cell death by apoptosis rather than necrosis. To identify which phase of the cell cycle the Jurkat cells were being apoptosed, the DNA content of the nucleus of each Jurkat cell was measured using flow cytometry (Section 2.2.5). Following treatment, the Jurkat cells were prepared for analysis by flow cytometry. Cell cycle analysis produced a histogram and a breakdown of the percentage of Jurkat cells in each phase of the cell cycle (Figure 3.3.1).

When the Jurkat cells were treated with 100 µM of each lignan compound for 4 h, there was no significant change in the percentage of cells fixed in G_1-phase or in S-phase compared to the untreated control (Figure 3.3.2 and Figure 3.3.3, respectively).
The percentage of Jurkat cells fixed in G_2-phase was significantly lower ($P<0.001$) following incubation with 100 µM of lignan compounds SD007, SD008, SD010, SD012, SD013, SD014, SD015, SD016 and SD018 for 4 hours when compared to the untreated control (Figure 3.3.4). Refer to the Appendices (Table 7.8) for absolute values.

As a positive control, the Jurkat cells were treated with 0.5 µM camptothecin for 4 hours in place of the lignan compounds. The percentage of Jurkat cells in G_1-phase was significantly increased compared to the untreated control (Figure 3.3.2). The mean percentage of Jurkat cells in S-phase was significantly decreased compared to the untreated control (Figure 3.3.3). The mean percentage of Jurkat cells fixed in G_2-phase were significantly decreased compared to the untreated control (Figure 3.3.4) Refer to the Appendices (Table 7.9) for absolute values.

To summarise, none of the lignan compounds showed any significant effect on the mean number of Jurkat cells in G_1-phase and S-phase, however lignan compounds SD007-8, SD010, SD012-16 and SD018 showed a significant decrease in the mean percentage of Jurkat cells in G_2-phase, which suggests the Jurkat cells are

undergoing cell death before reaching G_2-phase. Refer to the Appendices (Table 7.10) for absolute values.

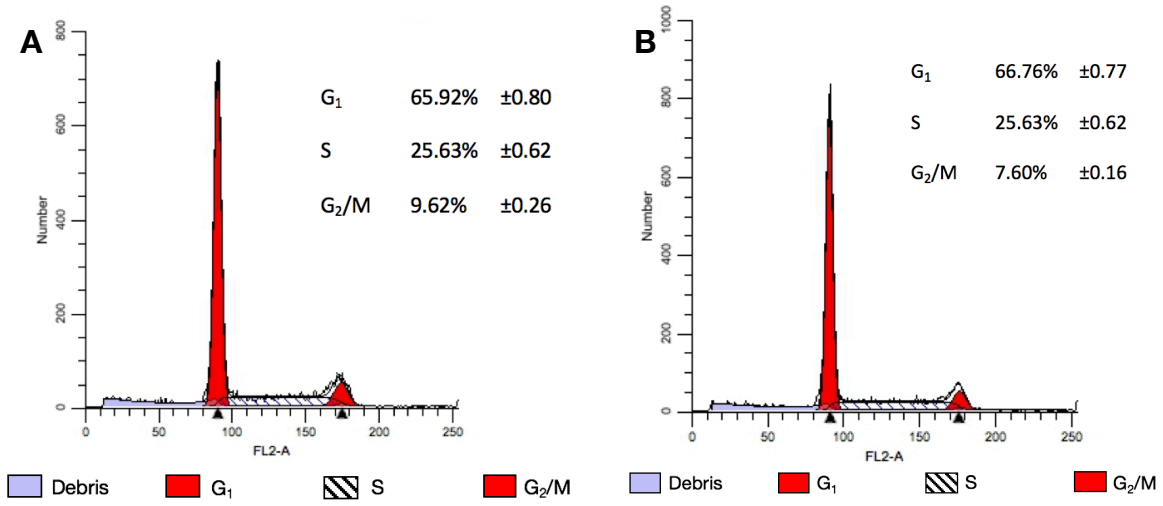


Figure 3.3.1: ***Cell cycle analysis of Jurkat cells following no treatment (A) and a 4-hour treatment with 100 µM SD007 (B), using ModFit LT software.*** *The histogram shows the number of Jurkat cells in G_1, S and G_2/M-phase measured by the area of fluorescence detected using the FL2 channel. A brief analysis of each sample is shown. A detailed analysis is also generated by the software, quantifying the number of cells in each phase (not pictured).*

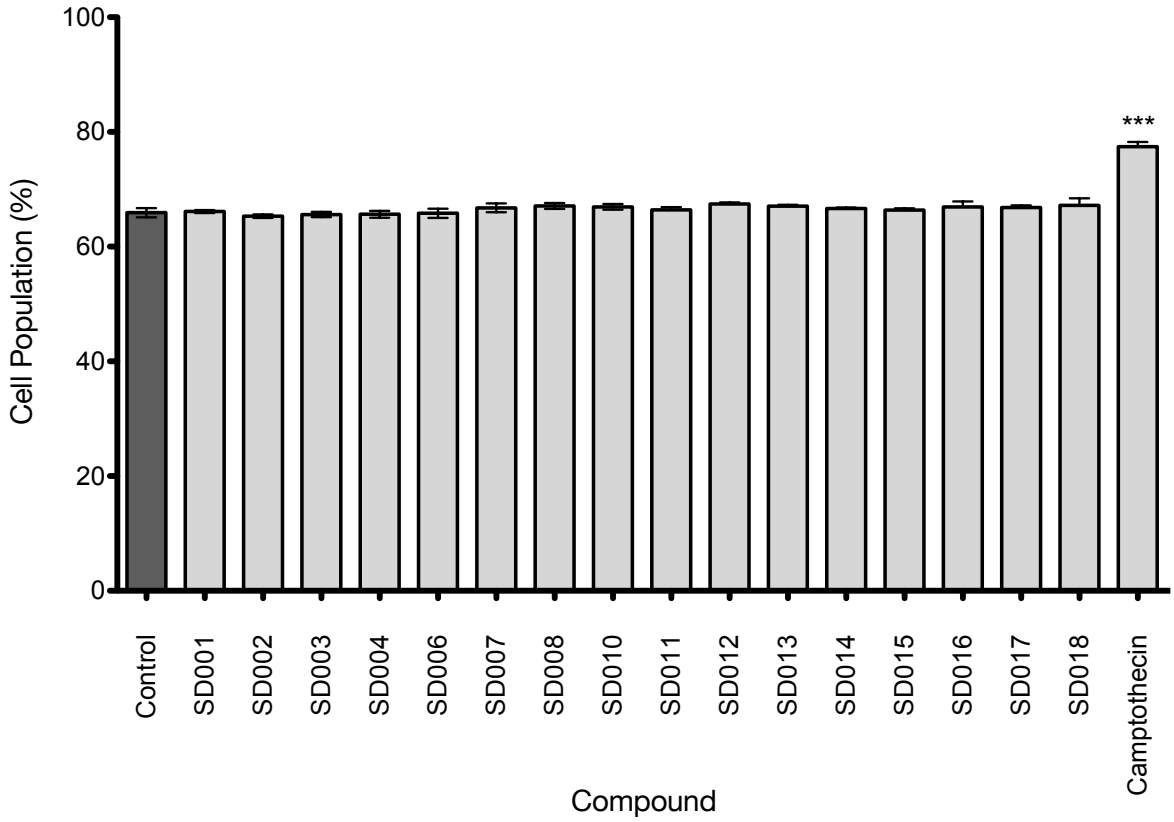


Figure 3.3.2: ***Mean percentage of Jurkat cells in G_1 following treatment for 4 hours with 100 μM of each lignan compound. As a positive control, Jurkat cells were treated with 0.5 μM camptothecin for 4 hours.*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. *** represents a p value < 0.001.*

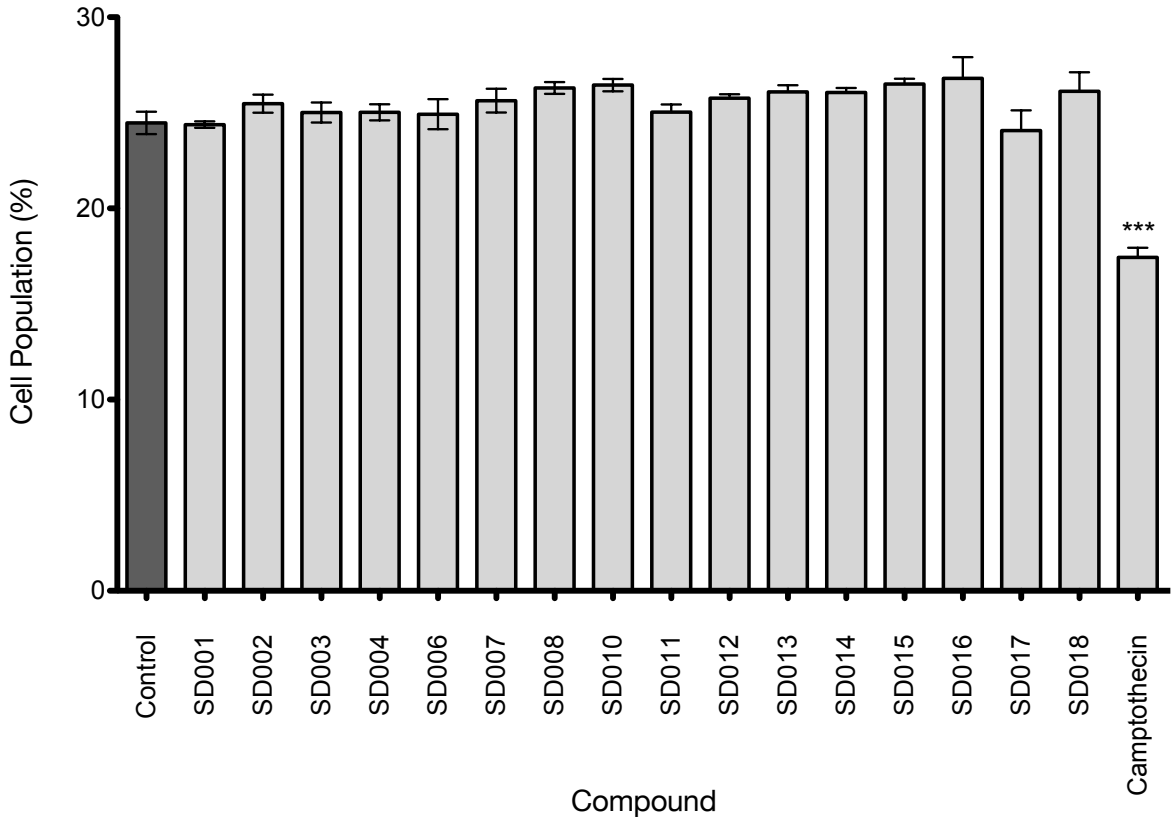


Figure 3.3.3: ***Mean percentage of Jurkat cells in S-phase following treatment for 4 hours with 100 μM of each lignan compound. As a positive control, Jurkat cells were treated with 0.5 μM camptothecin for 4 hours.*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. *** represents a p value < 0.001.*

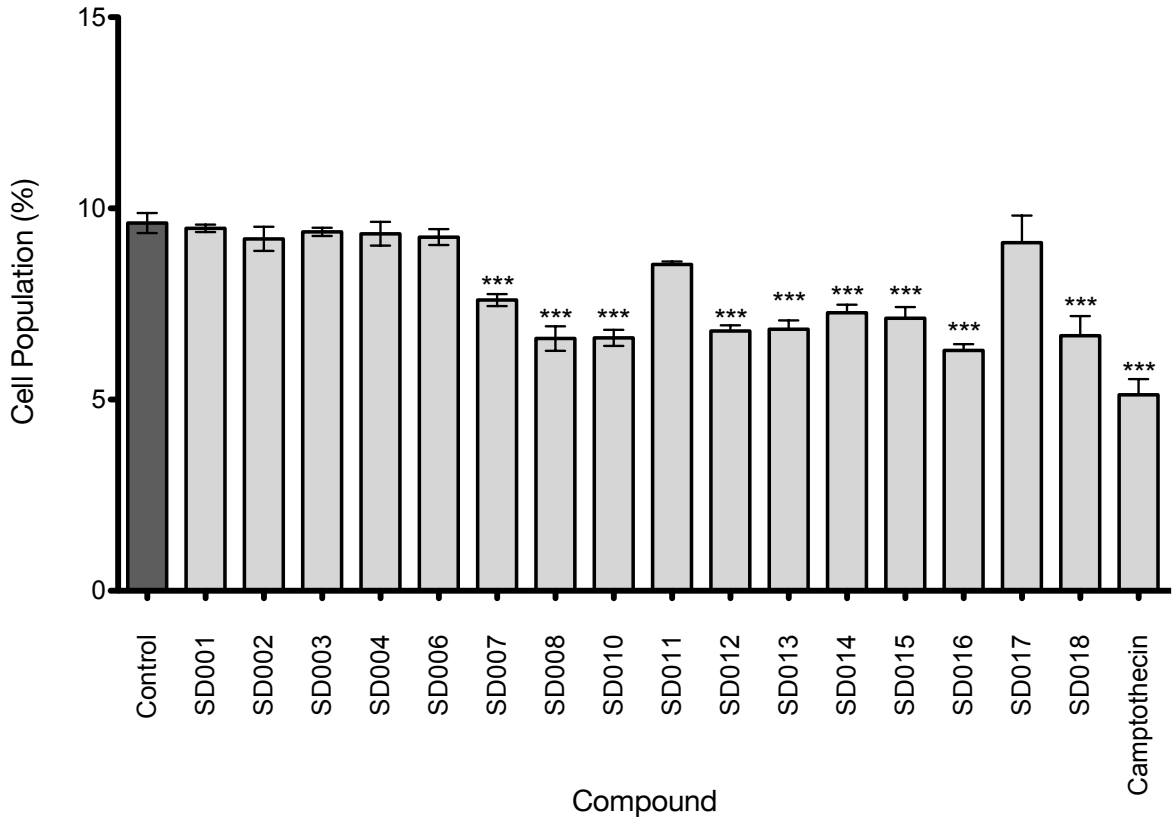


Figure 3.3.4: ***Mean percentage of Jurkat cells in G_2/M-phase following treatment for 4 hours with 100 μM of each lignan compound. As a positive control, Jurkat cells were treated with 0.5 μM camptothecin for 4 hours.*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. *** represents a p value < 0.001.*

3.4 Effect of novel lignan compounds on cell signalling and stress response in Jurkat cells following treatment

The Jurkat cells were treated with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018 for 4 hours.

Due to time constraints, a selection of lignan compounds were chosen for this investigation based on the results obtained from previous experiments (Sections 3.1-3.3). Lignan compounds SD007, SD010, SD012, SD013 and SD018 appeared to significantly increase the rate of apoptosis in the Jurkat cells following treatment, unlike SD011, which did not appear to have any significant pro-apoptotic effect on the Jurkat cells (Figure 3.2.4). Thus, comparing the results of the pro-apoptotic lignans on cell signalling and stress response with those of SD011 would indicate whether the Wnt/β-catenin or Akt/PI3K pathways are responsible for the pro-apoptotic properties of lignan compounds SD007, SD010, SD012, SD013 and SD018.

The lysates from the treated Jurkat cells were analysed for the presence of HSPB1, HSPA1A, Akt, phospho-Akt and phospho-β-catenin by Western Blotting.

When compared to the relative HSPB1 expression in the untreated control (1.00), there was a fold increase of 7.15, 2.11, 2.14, 2.17, 1.98 and 2.19 when the Jurkat cells were treated with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, respectively (Figures 3.4.1 and 3.4.2).

There was a fold decrease of 0.92 in relative HSPA1A expression compared to the untreated control (1.00) when treating the Jurkat cells with 100 μM of lignan compound SD007 for 4 h. Identical treatment with lignan compounds SD010, SD011, SD012, SD013 and SD018, demonstrated a fold increase of 1.44, 1.23, 18.25, 1.74 and 1.65 respectively (Figures 3.4.3 and 3.4.4).

Phospho-Akt levels were shown to decrease after a 4h treatment with 100 μM of lignan compounds SD007 (0.03), SD010 (0.90), SD011 (0.20), SD012 (0.02), SD013 (0.015) and SD018 (0.13) when compared to the untreated control (1.00; Figures 3.4.5 and 3.4.6).

When compared to the relative Akt expression in the untreated control (1.00), there was a fold increase of 1.52, 1.66, 1.23 and 1.01 when the Jurkat cells were treated

with 100 μM of lignan compounds SD007, SD010, SD011 and SD012, respectively. Identical treatment with lignan compounds SD013 and SD018 resulted in a fold decrease of 0.86 and 0.75, respectively (Figures 3.4.7 and 3.4.8).
Bands for phospho-β-catenin were not observed on the membrane following chemiluminescent detection (Figure 3.4.9).

In summary, the lignan compounds, particularly SD007, appeared to affect the expression of HSPB1, with a small increase in HSPA1A expression also observed. There was also a large fold increase in HSPA1A expression following treatment with lignan compound SD012. The effect of the lignan compounds on phospho-Akt, Akt and phospho-β-catenin expression however did not appear to change, suggesting a minimal effect on these cell signalling pathways.

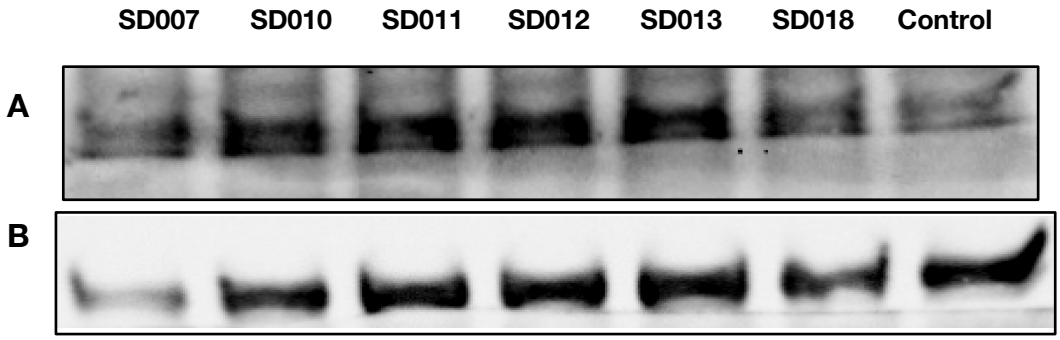


Figure 3.4.1: *Western Blot images of HSPB1 levels (A) and β-actin levels (B) using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

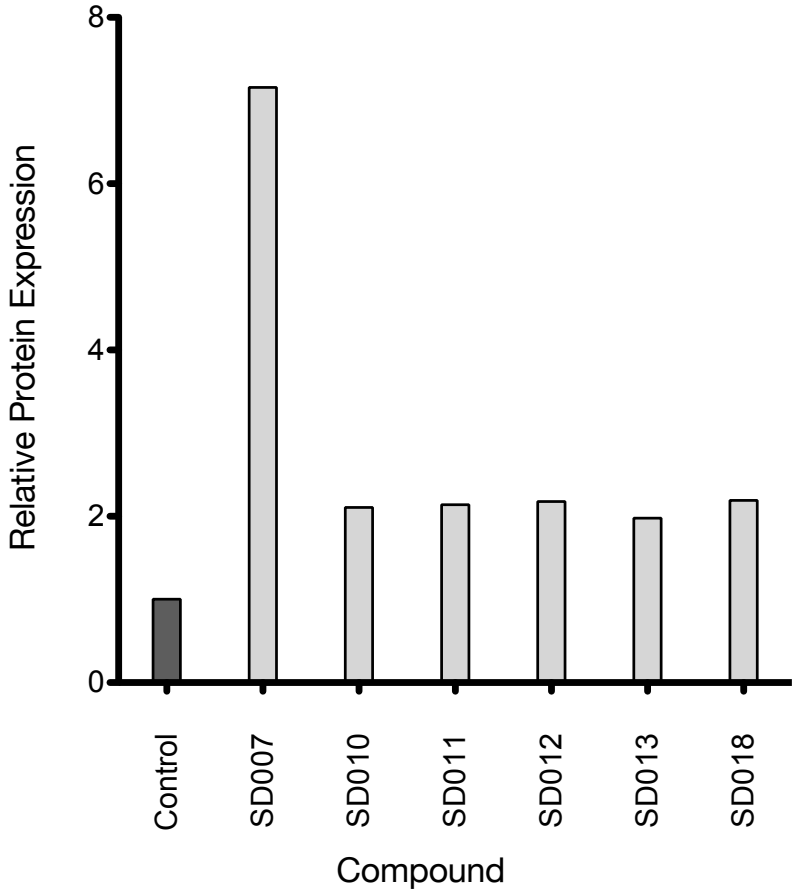


Figure 3.4.2: *Density results showing relative protein expression of HSPB1 in the lignan compound treated samples compared to the untreated control.*

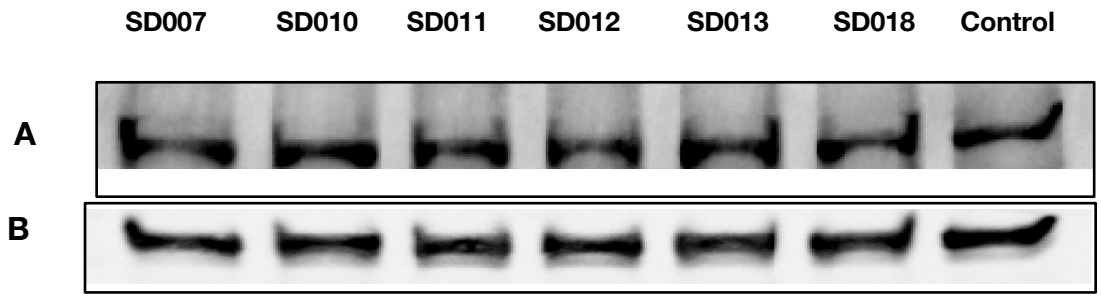


Figure 3.4.3: *Western Blot images of HSPA1A levels (A) and β-actin levels (B) using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

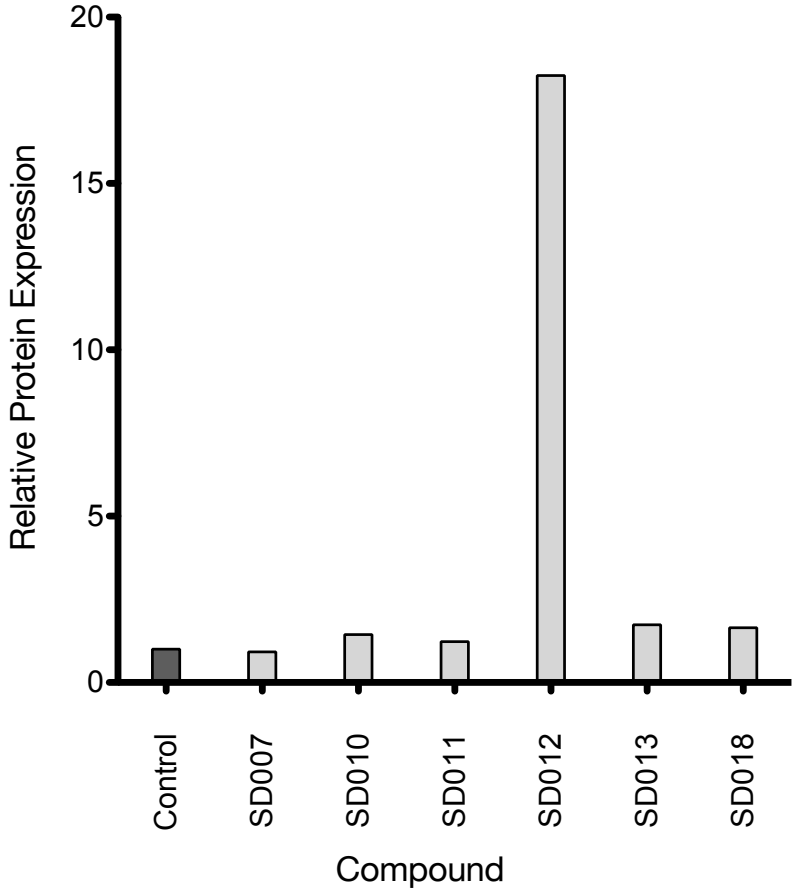


Figure 3.4.4: *Density results showing the relative protein expression of HSPA1A in the lignan compound treated samples compared to the untreated control.*

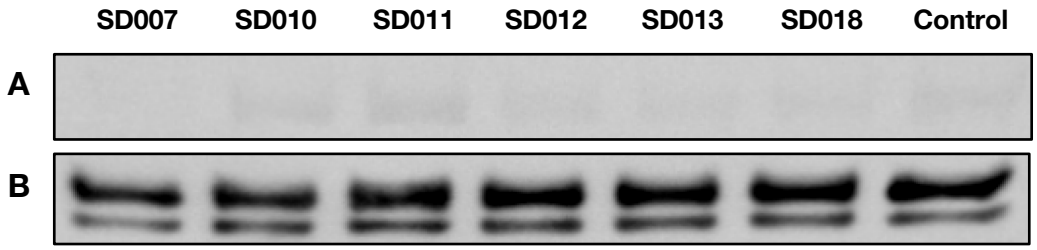


Figure 3.4.5: *Western Blot images of phospho-Akt levels (A) and β-actin levels (B) using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

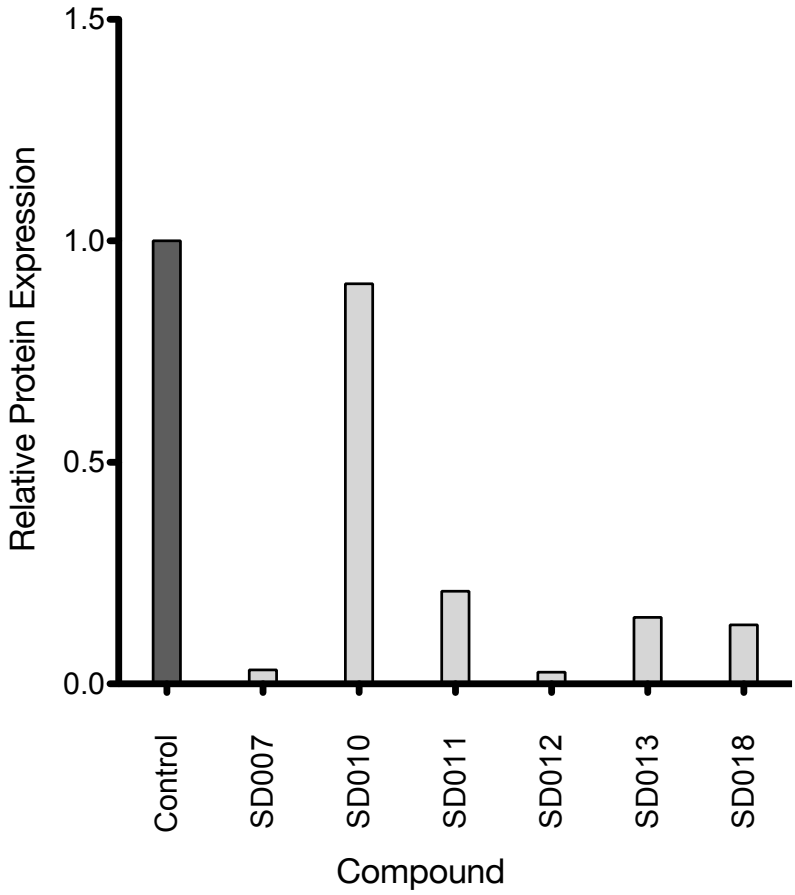


Figure 3.4.6: *Density results showing the relative protein expression of phospho-Akt in the lignan compound treated samples compared to the untreated control.*

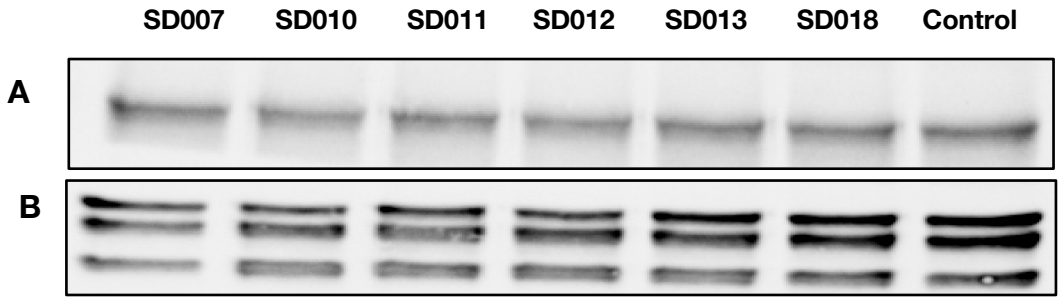


Figure 3.4.7: *Western Blot images of Akt levels (A) and β-actin levels (B) using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

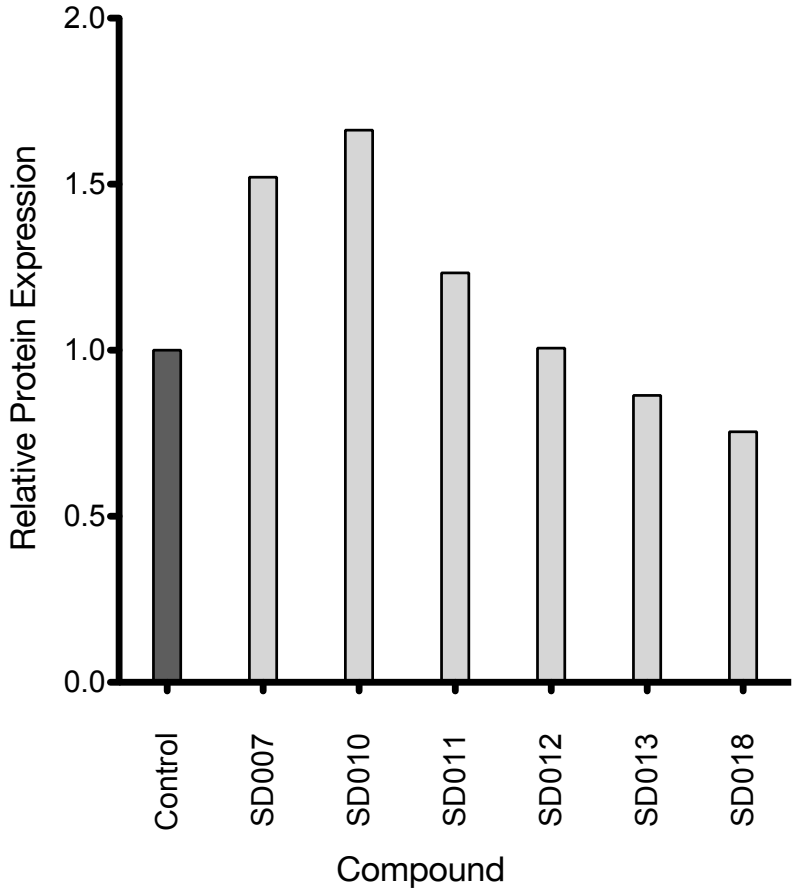


Figure 3.4.8: *Density results showing the relative protein expression of Akt in the lignan compound treated samples compared to the untreated control.*

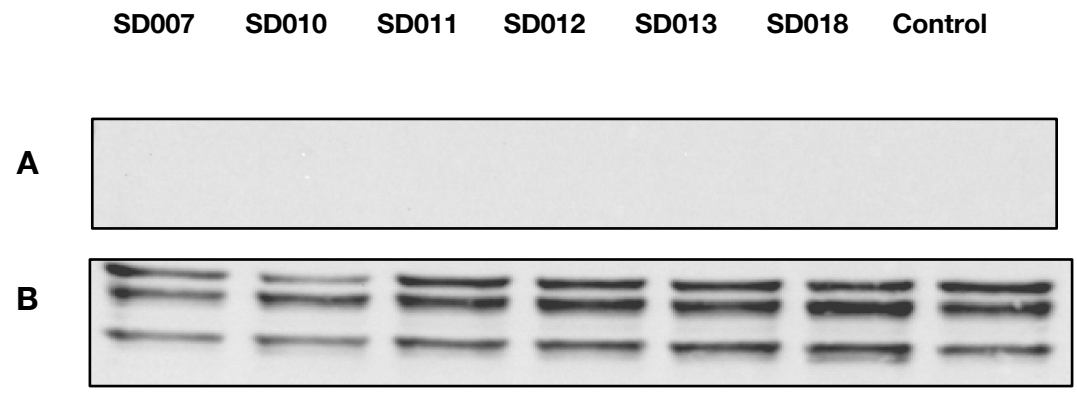


Figure 3.4.9: *Western Blot images of phospho-β-catenin levels (A) and β-actin levels (B) using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

4.0 Discussion

The purpose of the project was to identify whether the novel lignan compounds could have a role as anti-leukaemia agents. As previously mentioned, both natural and synthetic lignans have been described as possessing anti-cancer properties in *in-vitro* studies, in addition to well-established treatment regimens involving the chemotherapeutics etoposide and teniposide (Gordaliza et al., 2000). In order for new treatments to be licensed and used in recognised protocols, it is crucial that the full effects and mechanisms of the compounds are elucidated, the first stage of which involves assessing *in-vitro* effects on suitable cell models. The exact mode of cytotoxicity should be identified, as the pro-inflammatory effect of a necrosis-inducing drug could exacerbate collateral damage to non-tumour cells when compared to a relatively 'clean' pro-apoptotic treatment (Chandrashekar and Shobha Rani, 2008). Some potential chemotherapeutics may not induce cell death at all and could cause the cancer cells to enter a cytostatic state where further proliferation is inhibited, but the cells remain *in-situ* until removal by the innate immune system (Medon et al., 2017).

It is important to ensure that the new drug targets the cancer cell with minimal disruption or stresses to healthy tissues. While the treatment could be effective against the malignant cell, an undesirable effect towards the healthy vascular endothelium or gastrointestinal tract would not only affect the route of administration, but also the dosage required (Chandrashekar and Shobha Rani, 2008). Research surrounding the aberrant deregulation of signalling pathways in cancer cells, has highlighted that upstream and downstream components are a desirable target for novel anti-cancer therapies.

4.1 Investigating the effect of novel lignan compounds on cellular metabolism of Jurkat cells following treatment

MTS assays were used in this study to assess the effect of the novel lignan compounds on Jurkat biochemical activity. The metabolic activity of the Jurkat cells following treatment varied with each individual lignan compound.

Jurkat cell treatment with 200 μM of the lignan compounds SD002, SD006-10, and SD012-15 demonstrated a significant reduction in metabolic activity after a 24-hour

incubation. The same experiment also resulted in reduced metabolic activity after a 24-hour treatment with 100 μM of lignan compounds SD007 and SD013. This suggested that lignan compounds SD007 and SD013 were the most potent, as they significantly decreased cell metabolism at lower concentrations than the other lignan compounds. Following a 48-hour treatment with 200 μM of these same lignan compounds, there was a significant reduction in metabolic activity, with the exception of lignan compound SD015.

Treatment with 200 μM of lignan compounds SD001, SD003-5, SD011 and SD016-18 did not have a significant effect on the metabolic activity of the Jurkat cells after 24 hours, suggesting that these particular compounds were not effective anti-leukaemia agents. However, following a 48-hour incubation with the same lignan compounds, a significant decrease in metabolic activity was observed, highlighting the importance of including time-course experiments in such research.

Surprisingly, incubation of the Jurkat cells with lower concentrations of some lignan compounds (SD012 and SD013) resulted in an increase in metabolic activity when compared with the untreated cells, with this increase being statistically significant for some lignan compounds (Figure 3.1.2, Table 7.2 and Table 7.5). Cells may increase their rate of metabolism during cellular stress as they upregulate expression of key stress and anti-apoptotic genes and proteins (Jeon et al., 2011). These results highlight that even at low concentrations, some of the lignan compounds are exerting an effect on this cell model.

The data from the 48-hour time point suggests that treatment with 200 μM of all lignan compounds – with the exception of SD015 – significantly reduces metabolic activity. The data also supports the data from the 24-hour time point with regards to treatment with 100 μM of lignan compounds SD007 and SD013, where there is also a significant decrease in metabolic activity (Table 7.5).

One of the limitations of proliferation assays such as MTS, is that they are unable to distinguish between cytotoxicity and cytostasis. The principle of this colorimetric assay is that biochemically active cells will metabolise the tetrazolium compound to produce a formazan compound, which is directionally proportional to the absorbance of the solution at 450nm. Whether a cell has simply ceased proliferation or undergone cell death, biochemical reduction of the tetrazolium salt will cease.

One of the difficulties in comparing the present data with that from other published research is the frequent over-simplification of the published data. The MTT and MTS assays are often incorrectly described as cytotoxicity assays, suggesting that the assays are able to distinguish between cytotoxic and cytostatic cells. This means that many studies describe the anti-cancer chemotherapeutics under investigation as cytotoxic, despite cytostasis being the direct effect of the compound. An example of this is an article by Gordaliza et al. (2000), which incorrectly describes the cytostatics etoposide and teniposide as cytotoxic agents.

A study conducted by Yoo et al. (2010) using a colon cancer cell line (SW480) to investigate the effect of a 48 hours incubation with the natural lignans arctiin, matairesinol and arctigenin used a viability assay to analyse cell survival, albeit with a different product known as EZ-CyTox. This experiment showed that following a 48-hour incubation, cell survival was significantly decreased with 50 μM of each lignan. This experiment adds further weight to the possibility that a longer incubation time with the lignan compounds could influence cell viability. Additionally, a study by He et al. (2013) investigated the treatment of the colorectal cancer cell lines Caco-2, SW1116 and HCT-116 with the lignan wogonin at 0-200 μM for 6 hours, 12 hours and 24 hours using the MTT assay. However, unlike the study by Yoo et al. (2010), statistical analysis was not performed on this set of data therefore it is difficult to compare with other experiments.

The MTS assay data are particularly valuable as preliminary results, indicating not only which particular lignan compounds are the most effective at reducing biochemical activity, but also the concentration at which the lignan compounds may achieve this effect. The results highlighted lignan compounds SD002, SD006-10 and SD012-15 as the most potent treatments, with SD007 and SD013 showing a significant effect even at 100 μM.

4.2 Effect of lignan compounds on apoptosis and necrosis of Jurkat cells following treatment

The Annexin V/PI assay is a useful flow cytometric method that is able to quantify the number of viable, early apoptotic, late apoptotic and necrotic cells within a

sample. This technique elaborates on the data previously collected from the MTS assays, by using apoptosis and necrosis markers to indicate the mode of cell death. The Annexin V/PI assay confirms that cell death is occurring following treatment with some of the lignan compounds. The assay also suggests that the more effective lignan compounds do not act as cytostatic agents, but exhibit cytotoxic properties, owing to the presence of apoptotic and necrotic markers in the treated cells. The results indicated that lignan compounds SD007-10, SD012-16 and SD018 are of particular interest; however, each of these lignan compounds affect the Jurkat cells in different ways. For example, SD007 produced both an extremely vivid decrease in viable cells and increase in apoptosis only at a higher concentration of 200 μM and appeared to be much less potent after a 100 μM treatment (Figure 3.2.1 and 3.2.3C). However, lignan compounds SD012-14 and SD018 had a relatively modest but significant effect on Jurkat cell viability and apoptosis at both 200 μM and 100 μM (Figures 3.2.4D, 3.2.5A, 3.2.5B and 3.2.6B, respectively).

He et al. (2013) used the Annexin V/PI assay to examine the effect of the flavanoid wogonin on HCT-116 cells, albeit at the lower concentrations of 10 μM, 20 μM and 40 μM for 24 hours, which showed no significant alterations in the populations. The authors have also opted not to publish the exact method used to obtain the results of the assay, as it is only briefly mentioned in the caption of Figure 1 that the method is an Annexin V/PI stain. It is therefore difficult to compare this particular experiment with my own, as there is not enough information included in the article. There is also no mention in the study of why the lower concentrations of wogonin (10 μM, 20 μM and 40 μM) were chosen rather than higher concentrations.

A study published by Peuhu et al. (2013) investigating the inhibition of Akt signalling with the lignan matairesinol in a prostate cancer cell line (LNCaP) but did not use the Annexin V/PI assay as used in my study and in the study by He et al (2013). The method used by Peuhu et al. is traditionally used to quantify the amount of DNA in cells for cell cycle analysis, however sub-G_1 levels of DNA from apoptosed cells within the sample are also able to be detected. This is less specific than the Annexin V/PI assay used in my study for determination of apoptosis, as it is not possible to differentiate between early and late apoptosis with just the sub-G_1 levels of DNA

quantified in the samples, therefore it is difficult to compare the data between studies.

4.3 Effect of lignan compound treatment on the cell cycle of Jurkat cells following treatment

The flow cytometric cell cycle analysis assay is used to distinguish cells by quantifying the amount of DNA in each cell. The analysis gives an indicator as to the percentage of Jurkat cells that are in either G_1, S, or G_2/M-phase following lignan compound treatment.

The results from the assay provide some interesting details regarding the activity of the lignan compounds. None of the lignan compounds had any significant effect on the mean percentage of Jurkat cells in either G_1 or S-phase, however there was a significant decrease in the percentage of Jurkat cells in G_2/M-phase following treatment with lignan compounds SD007, SD008, SD010, SD012-16, and SD018. These particular lignan compounds were highlighted as potential pro-apoptotic agents in the Annexin V/PI assay. The significant reduction in Jurkat cells in G_2/M-phase adds further weight to the suggestion that following treatment with these lignan compounds in particular the cells undergo apoptosis either during or before mitotic division.

However, this data also appears to suggest that the lignan compounds do not affect the Wnt/β-catenin pathway, which plays an important role in the regulation of the cell cycle. The study conducted by Yoo et al. (2010) showed that a 24-hour treatment with 100 μM arctigenin on the SW480 cells induced a significant increase in G_2/M-phase cells and a significant decrease in G_1-phase cells. The same study demonstrated that the Wnt/β-catenin pathway was downregulated following a 100 μM arctigenin treatment, which does not correlate with the cell cycle data also published in the study by Yoo et al. Another project investigated the effect of a flavonoid on the Wnt/β-catenin pathway in HCT116 cells, and in contrast to the study by Yoo et al., demonstrated that the cells were arrested in G_1-phase, while also downregulating the activity of the Wnt/β-catenin pathway (He et al., 2013). Additionally, in another study, a G_1-phase arrest was achieved using a natural lignan in a breast cancer cell line (T47D) which also inhibited the Wnt-β-catenin pathway

downstream target cyclin D1 (Kim et al., 2010). It is important to note however, that Yoo et al., He at al. (2013), and Kim et al. (2010) incubated the cell lines with the treatments for up to 24 h, which is longer than the drug-exposure times used in the present, in which Jurkat cells, which were only incubated with the lignan compounds for 4 h. The research published by Peuhu et al. included cell cycle data, which also showed that the LNCaP cells were arrested in G_1-phase, which implies that there was no interference with the PI3K/Akt signalling cascade.

4.4 Effect of novel lignan compounds on cell signalling and stress response in Jurkat cells following treatment

Western Blotting was used to detect the presence of proteins of interest within lysates of the lignan compound treated Jurkat cells. Densitometry analysis was then used to calculate the fold change in protein expression compared with the untreated Jurkat cells.

The Western Blot and densitometry results for HSPB1 expression suggest that following a 4-hour incubation with 100 μM of lignan compound SD007, there was an approximately 7-fold increase in expression. There was also a 2-fold increase in HSPB1 following the same treatment with lignan compounds SD010, SD011, SD012, SD013 and SD018. The results for HSPA1A expression suggested relatively few changes following lignan treatments, with the exception of SD012, which appeared to induce a 17-fold increase in expression.

The densitometry results indicated there was relatively little fold change in Akt or p-Akt expression, if any. Also, no bands were observed during Western Blot detection for p-β-catenin.

Increased HSPB1 expression after treatment with the lignan compounds suggests the Jurkat cells were undergoing a cellular stress response to the lignan treatment. One of the functions of HSPB1 is to inactivate the caspase cascade by binding with caspase-3 and cytochrome *c*, thus inhibiting the apoptotic pathway (Wang et al., 2014; Acunzo et al., 2012). HSPB1 is therefore highly induced in cells undergoing a survival response to cytotoxic stimuli. This increased HSPB1 level suggests that lignan compound SD007 is inducing apoptosis in the Jurkat cells and that the cells are responding by upregulating HSPB1 to ensure cell survival. Lignan compound

SD007 shows the largest fold increase, which is consistent with the Annexin V/PI data, where the lignan compound induced significant apoptosis at 200 μM. It would be interesting to investigate the HSPB1 levels after longer time points following lignan treatment, as the Annexin V/PI data indicates apoptotic activity despite the strong induction of HSPB1 in the cells. It is important to note that Western Blot analysis was only performed on lysates from Jurkat cells treated at 100 μM concentrations for 4 hours. However, the striking effects of this lignan in comparison with others when considering the MTS and Annexin V/PI data make it plausible to assume that HSPB1 may also be strongly induced following higher concentrations of the compound. The 17-fold increase in HSPA1A expression following SD012 treatment is an interesting result. Although SD012 was an effective apoptotic agent at 100 μM in previous experiments (Figure 3.2.4D), the result is not entirely consistent as lignan compounds SD013, SD014 and SD018 returned similar results following the Annexin V/PI assay (Section 3.2) however did not demonstrate HSPA1A induction. It is possible that SD012 is a much stronger HSPA1A inducer than other lignan compounds, however further examination is necessary to elucidate this. The result could also be attributed to the uneven shape of the bands, leading to them not being measured correctly during densitometry analysis. Unfortunately, there is limited literature investigating the effects of lignans on heat shock protein expression in cancer. In contrast to the experimental data, a study investigating the effect of arctigenin on the heat shock response showed that a 3 hour incubation of cervical cancer cells (HeLa) with 100 μM arctigenin suppressed the expression of HSPA1A (Ishihara et al., 2006). Another comparable study investigated the effect of the oral administration of the lignan schisandrin B on HSPB1 and HSPA1A expression in mice with hepatic injury. This study showed a significant increase in the expression of both HSPB1 and HSPA1A (Li et al., 2014).

The anti-apoptotic properties of HSPB1 and HSPA1A have been strongly associated with chemotherapy resistance in cancer cells (Wang et al., 2014). Yamamoto et al. (2001) compared the levels of HSPB1 and HSPA1A between a cisplatin resistant and cisplatin sensitive ovarian cancer cell line and identified upregulated HSPB1 and HSPA1A in the cisplatin resistant cells. Increased HSPA1A levels have also been observed in relapsed CML patients that are refractory to imatinib mesylate (Zorzi &

Bonvini, 2011). These studies suggest that the increased HSPB1 expression observed in the Jurkat cells following lignan compound treatment, especially when treated with SD007, could be associated with chemotherapy resistance, which could mean any surviving Jurkat cells could become resistant to future courses of treatment.

The relatively subtle changes in expression of p-Akt and Akt suggest that the lignan compounds do not affect the PI3K/Akt pathway. Further evidence is the cell cycle analysis (Section 4.3), which did not show an increase in the number of cells arrested in G_1-phase as a previous study by Peuhu et al. where Akt signalling decreased in response to treatment of LNCaP cells by the natural lignan matairesinol. However, as LNCaP cells are a prostate carcinoma cell line and matairesinol is a different lignan, these could be legitimate reasons for the differences in data. Additionally, the same research group identified the lignan nortrachelogenin as possessing the same properties *in-vitro* (Peuhu et al., 2013).

The absence of bands on the Western Blot probed for p-β-catenin, in addition to the aforementioned cell cycle data (Section 4.3), indicates that the Wnt/β-catenin pathway is not affected by the lignan compounds. The study by Yoo et al., which described regulation of the Wnt/β-catenin pathway following arctigenin treatment, used SW480 cells rather than Jurkat which could explain the contrasting results. Additionally, as mentioned in Section 4.3, the cell cycle data conflicted with other published papers, as well as the vague descriptions of the results within the article by the authors.

4.5 Future Work

The experiments conducted have indicated that the lignan compounds show promise as anti-cancer agents, in particular lignan compounds SD007, SD013 and SD018. It is interesting to note, that these three lignan compounds were the only compounds which did not contain a methyl (MeO) group at any of the molecules side chains.

It would be useful to perform additional cell cycle analysis could be performed to determine the effects of the lignans at different concentrations, as well as at following

longer treatment durations. Yoo et al., He at al., and Kim et al. obtained significant data by incubating the cells with the polyphenolic compounds for 24 hour before performing cell cycle analysis, which suggests this could be an interesting time point to observe changes in cell cycle. It would also be useful to perform Western Blots on lysates at this same 24 hour time point. However it was difficult to extract protein from Jurkat cells treated with lignan compound SD007, as the cells were undergoing apoptosis before protein was extracted. Conversely, in the study of phosphorylation events, it may be beneficial to reduce the treatment duration of cells in order to capture these events which can happen within minutes, before de-phosphorylation of the specific kinase occurs and the window for detection is missed.

Performing quantitative polymerase chain reaction (qPCR) using mRNA collected from lignan compound-treated cells in future work would allow detailed examination of the effects of these compounds on key signalling and stress response genes, which would support protein analysis work by the Western Blot. For example, it would be interesting to look at HSPA1A and HSPB1 gene expression after lignan compound treatment, which could confirm the effect of the lignan treatment on HSPB1 and HSPA1A expression. It would also be beneficial to investigate the expression of target genes involved in cell signalling pathways.

In addition to the Wnt/β-catenin and PI3K/Akt pathways investigated in this thesis, other pathways that could be of interest are the NOTCH pathway, which is heavily implicated in T-cell development, as well as the JAK/STAT pathway (Willis, 2011; Zhou et al., 2013).

Additional *in-vitro* studies could be conducted with other leukaemia cell lines, such as B-cell malignancies, as well as primary cells obtained from patients with leukaemia would be a useful step in the pre-clinical development of the compounds. When considering moving any compound forward into *in-vivo* studies, the effects of *in-vivo* metabolism on the activity of the compound need consideration. Many compounds may be metabolised by the liver into products that have differing activity from that of the original compound. While still in the *in-vitro* stage of testing, liver microsomes can be used as a source of hepatic enzymes to replicate *in-vivo* breakdown of the compound. Future work investigating the metabolism of these lignan compounds using liver microsomes would be extremely beneficial. Gut flora

metabolism of compounds is an extra consideration when working with lignans. Natural lignans are metabolised by gut microflora to produce enterodiol and enterolactone (Section 1.4), and this may differ with synthetic lignans. Therefore, experiments involving *in-vitro* metabolism of the compounds using specific gut microflora species would also be useful.

5.0 Conclusion

The anti-leukaemia potential of the novel lignan compounds were explored, using viability and apoptosis assays, analysis of cell cycle distribution and analysis of key protein expression. The results indicated that the lignan compounds, SD007, SD008, SD010, SD012-16 and SD018 demonstrated pro-apoptotic properties, with SD007, SD013 and SD018 of particular interest.

The data suggested that the lignan compounds had no effect on the regulation of the Wnt/β-catenin and PI3K/Akt pathways, thus the effect of the lignan compounds on the cell signalling cascades remain elusive.

The increase of HSPB1 expression following lignan treatment suggested that the Jurkat cells were undergoing a cellular stress response which could eventually lead to the development of treatment resistance, particularly following treatment with lignan compound SD007.

Subsequent work with these compounds would aim to identify the mechanisms by which the lignan compounds induce apoptosis, and to further establish the cellular stress induced by the lignan compounds. Despite inducing apoptosis, the induction of HSPB1 and HSPA1A by some lignans highlights the need for further research to elucidate the usefulness of the lignan compounds.

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7.0 Appendices

Table 7.1: Absolute data from MTS assay of Jurkat cells treated with 0-200 µM lignan compounds SD001-18 for 24 hours.

SD001	Metabolic Activity (%)			SD002	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	85.78024	2.482796	3	200.000	82.5023	6.770195	3
100.000	105.7479	4.211288	2	100.000	93.53646	4.501556	3
50.000	119.2059	13.32042	3	50.000	103.6011	3.13398	2
25.000	102.3546	10.06136	3	25.000	107.6177	0.7835007	2
12.500	106.2788	6.751755	3	12.500	107.964	2.252563	2
0.000	100.0693	11.5026	6	0.000	100.0693	11.5026	6

SD003	Metabolic Activity (%)			SD004	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	95.57178	4.355972	3	200.000	94.2579	4.862274	3
100.000	102.1411	4.722206	3	100.000	112.0681	10.68423	3
50.000	138.3212	8.154938	2	50.000	122.0438	5.448593	3
25.000	114.9392	8.331841	3	25.000	117.9562	4.386871	3
12.500	112.9927	10.0157	3	12.500	113.9659	5.829056	3
0.000	99.95134	7.302589	6	0.000	99.95134	7.302589	6

SD005	Metabolic Activity (%)			SD006	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	99.70971	3.694611	2	200.000	79.86668	7.009827	3
100.000	122.5447	10.72874	3	100.000	107.3333	4.291595	3
50.000	169.6662	6.362915	2	50.000	125.5556	6.32502	3
25.000	159.5065	3.694641	2	25.000	103.4222	11.30531	3
12.500	183.0188	35.50929	2	12.500	109.200	17.91337	2
0.000	100.0242	19.01103	6	0.000	99.95556	3.753914	6

SD007	Metabolic Activity (%)			SD008	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	-15.06667	2.887522	3	200.000	80.07053	5.524315	3
100.000	54.46667	6.693943	2	100.000	88.78307	2.805725	3
50.000	86.80001	11.86817	3	50.000	94.5679	5.398216	3
25.000	89.91111	4.636734	3	25.000	108.1834	8.847427	3
12.500	109.0222	17.12559	3	12.500	110.6526	1.165634	3
0.000	99.95556	3.753914	6	0.000	100.0353	8.432381	6

SD009	Metabolic Activity (%)			SD010	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	72.28187	8.950813	3	200.000	63.77424	7.037109	3
100.000	87.40711	2.744339	2	100.000	87.51323	14.02386	3
50.000	93.10488	1.459755	2	50.000	81.19928	6.930481	3
25.000	120.6854	9.75119	2	25.000	95.2028	10.1114	3
12.500	130.0578	0.7006989	2	12.500	104.2681	9.629234	3
0.000	99.97249	11.29295	6	0.000	100.0353	8.432381	6

SD011	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	122.4745	5.651497	3
100.000	119.1827	13.73535	3
50.000	134.0522	10.78079	3
25.000	140.4843	7.137756	3
12.500	149.4892	16.44527	3
0.000	99.97729	17.16466	5

SD012	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	70.0604	1.646133	3
100.000	97.87173	3.458076	3
50.000	100.0575	4.211311	3
25.000	102.646	1.91835	3
12.500	119.7584	2.92849	2
0.000	99.98562	10.30106	6

SD013	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	57.37705	4.758778	2
100.000	69.28386	6.83313	2
50.000	81.10439	3.760918	3
25.000	98.15932	7.495224	3
12.500	124.2019	10.18869	2
0.000	99.98562	10.30106	6

SD014	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	80.07108	3.940079	3
100.000	94.84641	5.349411	3
50.000	105.7883	4.880867	3
25.000	110.0787	1.382133	3
12.500	105.3821	3.155273	3
0.000	99.96192	9.784386	6

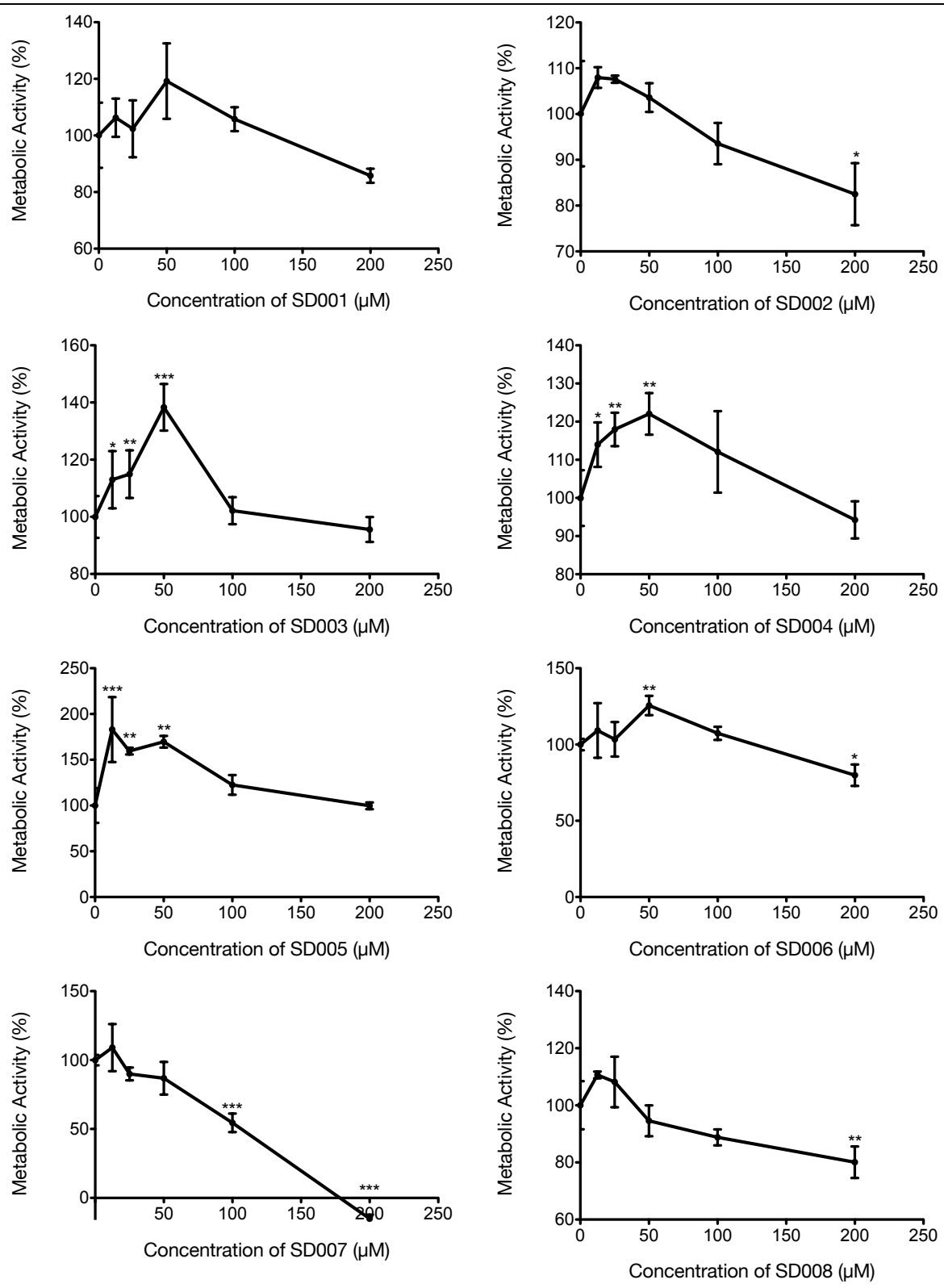
SD015	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	81.34045	1.507919	2
100.000	89.97206	4.233871	3
50.000	92.99316	3.000412	3
25.000	98.12136	1.088684	3
12.500	102.4372	2.369583	2
0.000	99.96192	9.784386	6

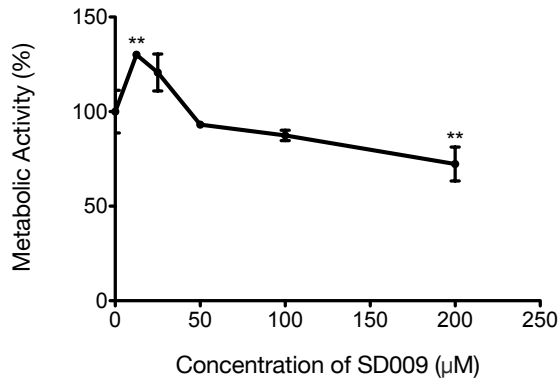
SD016	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	90.6094	0.706398	2
100.000	86.97969	6.206963	3
50.000	103.9627	4.494751	3
25.000	125.9407	16.31045	3
12.500	116.2504	8.992287	3
0.000	100.000	12.37921	6

SD017	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	93.05695	8.406166	2
100.000	90.94241	5.954178	3
50.000	98.4349	5.635582	3
25.000	93.7729	1.272827	3
12.500	88.14518	11.98566	3
0.000	100.000	12.37921	6

SD018	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	129.9175	4.960449	2
100.000	161.0271	10.87123	3
50.000	160.2476	3.784561	3
25.000	171.0683	12.14384	3
12.500	177.7167	13.28207	3
0.000	100.000	55.05859	6

Table 7.2: ***Graphs from 24 hour MTS Assays for Lignan Compounds SD001-18.*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*

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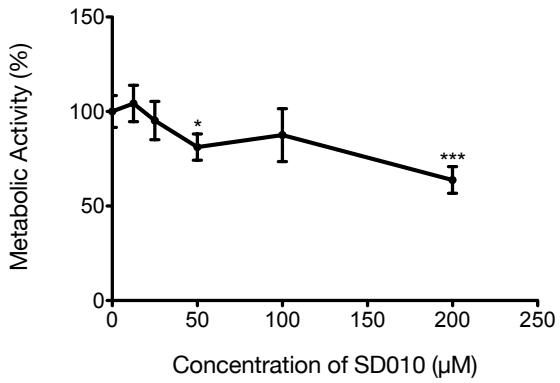
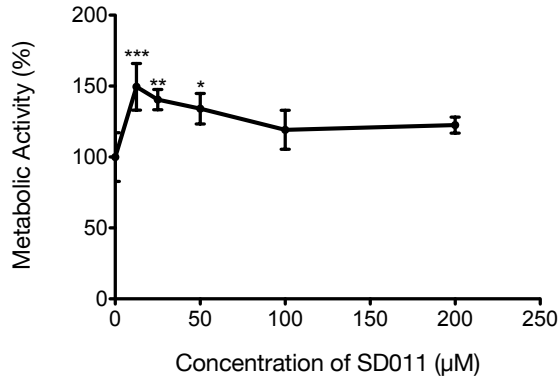
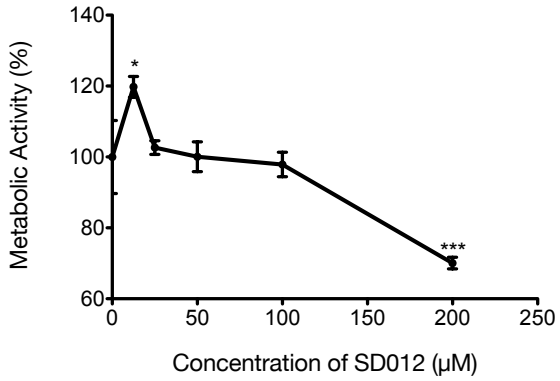
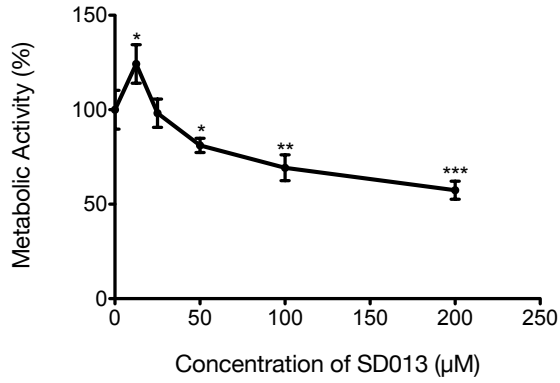
100
50
0
0
50
100
150
200
250
**
**
Concentration of SD009 (μM)


Metabolic Activity (%)
150
100
50
0
0
50
100
150
200
250
*
***
Concentration of SD010 (μM)


Metabolic Activity (%)
200
150
100
50
0
0
50
100
150
200
250
***
**
*
Concentration of SD011 (μM)


Metabolic Activity (%)
140
120
100
80
60
0
50
100
150
200
250
*
***
Concentration of SD012 (μM)


Metabolic Activity (%)
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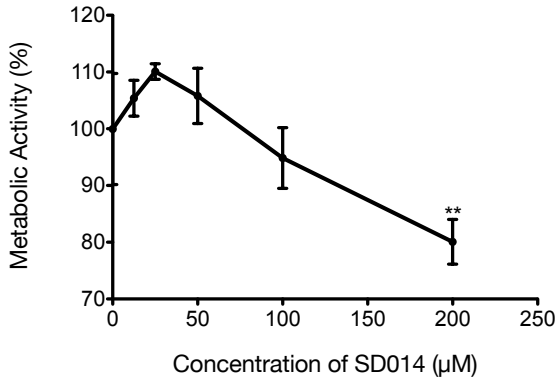
100
50
0
0
50
100
150
200
250
*
*
**
***
Concentration of SD013 (μM)


Metabolic Activity (%)
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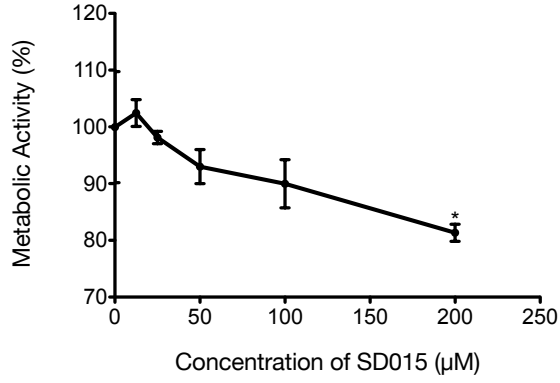
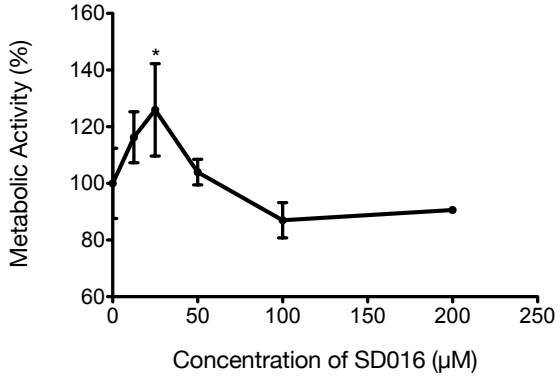
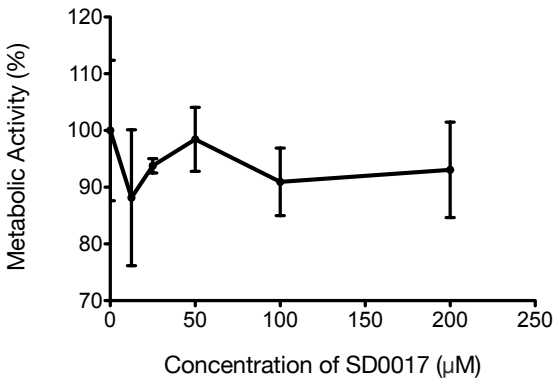
100
90
80
70
0
50
100
150
200
250
**
Concentration of SD014 (μM)


Metabolic Activity (%)
120
110
100
90
80
70
0
50
100
150
200
250
*
Concentration of SD015 (μM)


Metabolic Activity (%)
160
140
120
100
80
60
0
50
100
150
200
250
*
Concentration of SD016 (μM)


Metabolic Activity (%)
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110
100
90
80
70
0
50
100
150
200
250
Concentration of SD0017 (μM)

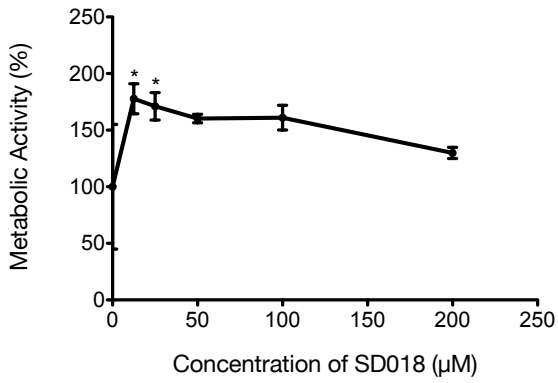

Metabolic Activity (%)
250
200
150
100
50
0
*
*
0
50
100
150
200
250
Concentration of SD018 (μM)

Table 7.3: *One-Way ANOVAs with Dunnett's post hoc test for* absolute data from MTS assay of Jurkat cells treated with 0-200 µM lignan compounds SD001-18 for 24 hours.

Table Analyzed	SD001				
One-way analysis of variance					
P value	0.0240				
P value summary	*				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	3.709				
R squared	0.5698				
ANOVA Table	SS	df	MS		
Treatment (between columns)	1775	5	355.0		
Residual (within columns)	1340	14	95.72		
Total	3115	19			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	14.29	2.065	No	ns	-5.359 to 33.94
Column F vs Column B	-5.679	0.710 9	No	ns	-28.37 to 17.01
Column F vs Column C	-19.14	2.766	No	ns	-38.78 to 0.5117
Column F vs Column D	-2.285	0.330 3	No	ns	-21.93 to 17.36
Column F vs Column E	-6.210	0.897 6	No	ns	-25.86 to 13.44

Table Analyzed	SD002				
One-way analysis of variance					
P value	0.0291				
P value summary	*				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	3.713				
R squared	0.6074				
ANOVA Table	SS	df	MS		
Treatment (between columns)	1252	5	250.4		
Residual (within columns)	809.3	12	67.44		
Total	2061	17			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	17.57	3.025	Yes	*	0.7196 to 34.41
Column F vs Column B	6.533	1.125	No	ns	-10.31 to 23.38
Column F vs Column C	-3.532	0.526 7	No	ns	-22.99 to 15.92
Column F vs Column D	-7.548	1.126	No	ns	-27.00 to 11.91
Column F vs Column E	-7.895	1.177	No	ns	-27.35 to 11.56

Table Analyzed	SD003				
One-way analysis of variance					
P value	0.0002				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	11.16				
R squared	0.7995				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3011	5	602.2		
Residual (within columns)	755.2	14	53.94		
Total	3766	19			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	4.380	0.843	No	ns	-10.37 to 19.13
Column F vs Column B	-2.190	0.421	No	ns	-16.94 to 12.56
Column F vs Column C	-38.37	6.399	Yes	***	-55.40 to -21.34
Column F vs Column D	-14.99	2.886	Yes	*	-29.74 to -0.2385
Column F vs Column E	-13.04	2.511	No	ns	-27.79 to 1.708

Table Analyzed	SD004				
One-way analysis of variance					
P value	0.0006				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	8.445				
R squared	0.7379				
ANOVA Table	SS	df	MS		
Treatment (between columns)	1993	5	398.6		
Residual (within columns)	708.0	15	47.20		
Total	2701	20			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	5.693	1.172	No	ns	-7.988 to 19.37
Column F vs Column B	-12.12	2.494	No	ns	-25.80 to 1.565
Column F vs Column C	-22.09	4.548	Yes	**	-35.77 to -8.411
Column F vs Column D	-18.00	3.706	Yes	**	-31.69 to -4.323
Column F vs Column E	-14.01	2.885	Yes	*	-27.70 to -0.3331

Table Analyzed	SD005				
One-way analysis of variance					
P value	0.0004				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	11.69				
R squared	0.8416				
ANOVA Table	SS	df	MS		
Treatment (between columns)	17880	5	3577		
Residual (within columns)	3366	11	306.0		
Total	21250	16			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	0.3145	0.02202	No	ns	-41.70 to 42.32
Column F vs Column B	-22.52	1.821	No	ns	-58.90 to 13.86
Column F vs Column C	-69.64	4.876	Yes	**	-111.7 to -27.63
Column F vs Column D	-59.48	4.165	Yes	**	-101.5 to -17.47
Column F vs Column E	-82.99	5.811	Yes	***	-125.0 to -40.98

Table Analyzed	SD006				
One-way analysis of variance					
P value	0.0002				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	10.78				
R squared	0.7938				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3319	5	663.8		
Residual (within columns)	862.1	14	61.58		
Total	4181	19			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	20.09	3.620	Yes	*	4.330 to 35.85
Column F vs Column B	-7.378	1.330	No	ns	-23.14 to 8.381
Column F vs Column C	-25.60	4.614	Yes	**	-41.36 to -9.841
Column F vs Column D	-3.467	0.6248	No	ns	-19.23 to 12.29
Column F vs Column E	-9.244	1.443	No	ns	-27.44 to 8.953

Table Analyzed	SD007

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	89.78				
R squared	0.9698				
ANOVA Table	SS	df	MS		
Treatment (between columns)	33450	5	6690		
Residual (within columns)	1043	14	74.52		
Total	34500	19			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	115.0	18.84	Yes	***	97.69 to 132.4
Column F vs Column B	45.49	6.454	Yes	***	25.47 to 65.51
Column F vs Column C	13.16	2.155	No	ns	-4.180 to 30.49
Column F vs Column D	10.04	1.646	No	ns	-7.291 to 27.38
Column F vs Column E	-9.067	1.485	No	ns	-26.40 to 8.269

Table Analyzed	SD008
One-way analysis of variance	
P value	0.0003
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	9.533
R squared	0.7606

ANOVA Table	SS	df	MS
Treatment (between columns)	2065	5	413.0
Residual (within columns)	649.9	15	43.32
Total	2715	20	

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	19.96	4.290	Yes	**	6.857 to 33.07
Column F vs Column B	11.25	2.418	No	ns	-1.855 to 24.36
Column F vs Column C	5.467	1.175	No	ns	-7.640 to 18.57
Column F vs Column D	-8.148	1.751	No	ns	-21.26 to 4.959
Column F vs Column E	-10.62	2.281	No	ns	-23.72 to 2.490

Table Analyzed	SD009

One-way analysis of variance					
P value	0.0003				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	13.04				
R squared	0.8557				
ANOVA Table	SS	df	MS		
Treatment (between columns)	5354	5	1071		
Residual (within columns)	903.1	11	82.10		
Total	6258	16			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	27.69	4.322	Yes	**	8.845 to 46.54
Column F vs Column B	12.57	1.698	No	ns	-9.195 to 34.33
Column F vs Column C	6.868	0.928 3	No	ns	-14.89 to 28.63
Column F vs Column D	-20.71	2.800	No	ns	-42.47 to 1.048
Column F vs Column E	-30.09	4.067	Yes	**	-51.85 to -8.325

Table Analyzed	SD010				
One-way analysis of variance					
P value	0.0007				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	8.116				
R squared	0.7301				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3609	5	721.7		
Residual (within columns)	1334	15	88.93		
Total	4943	20			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	36.26	5.438	Yes	***	17.48 to 55.04
Column F vs Column B	12.52	1.878	No	ns	-6.257 to 31.30
Column F vs Column C	18.84	2.825	Yes	*	0.05737 to 37.61
Column F vs Column D	4.832	0.724 7	No	ns	-13.95 to 23.61
Column F vs Column E	-4.233	0.634 8	No	ns	-23.01 to 14.55

Table Analyzed	SD011

One-way analysis of variance					
P value	0.0021				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	6.751				
R squared	0.7068				
ANOVA Table	SS	df	MS		
Treatment (between columns)	6015	5	1203		
Residual (within columns)	2495	14	178.2		
Total	8510	19			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	-22.50	2.308	No	ns	-50.19 to 5.191
Column F vs Column B	-19.21	1.970	No	ns	-46.89 to 8.483
Column F vs Column C	-34.07	3.495	Yes	*	-61.76 to -6.386
Column F vs Column D	-40.51	4.155	Yes	**	-68.20 to -12.82
Column F vs Column E	-49.51	5.079	Yes	***	-77.20 to -21.82

Table Analyzed	SD012				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	15.51				
R squared	0.8471				

ANOVA Table	SS	df	MS
Treatment (between columns)	3386	5	677.2
Residual (within columns)	611.3	14	43.66
Total	3997	19	

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	29.93	6.405	Yes	***	16.65 to 43.20
Column F vs Column B	2.114	0.4524	No	ns	-11.16 to 15.38
Column F vs Column C	-0.07190	0.01539	No	ns	-13.34 to 13.20
Column F vs Column D	-2.660	0.5694	No	ns	-15.93 to 10.61
Column F vs Column E	-19.77	3.665	Yes	*	-35.10 to -4.450

Table Analyzed	SD013

One-way analysis of variance	
P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	18.02
R squared	0.8825

ANOVA Table	SS	df	MS
Treatment (between columns)	6341	5	1268
Residual (within columns)	844.4	12	70.36
Total	7185	17	

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	42.61	6.221	Yes	***	22.74 to 62.48
Column F vs Column B	30.70	4.483	Yes	**	10.83 to 50.57
Column F vs Column C	18.88	3.183	Yes	*	1.673 to 36.09
Column F vs Column D	1.826	0.307 9	No	ns	-15.38 to 19.04
Column F vs Column E	-24.22	3.536	Yes	*	-44.09 to -4.345

Table Analyzed	SD014
One-way analysis of variance	
P value	0.0006
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	8.256
R squared	0.7335

ANOVA Table	SS	df	MS
Treatment (between columns)	1757	5	351.4
Residual (within columns)	638.3	15	42.56
Total	2395	20	

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	19.89	4.312	Yes	**	6.900 to 32.88
Column F vs Column B	5.116	1.109	No	ns	-7.875 to 18.11
Column F vs Column C	-5.826	1.263	No	ns	-18.82 to 7.164
Column F vs Column D	-10.12	2.193	No	ns	-23.11 to 2.874
Column F vs Column E	-5.420	1.175	No	ns	-18.41 to 7.570

Table Analyzed	SD015

One-way analysis of variance					
P value	0.0296				
P value summary	*				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	3.578				
R squared	0.5792				
ANOVA Table	SS	df	MS		
Treatment (between columns)	747.0	5	149.4		
Residual (within columns)	542.8	13	41.75		
Total	1290	18			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	18.62	3.530	Yes	*	3.490 to 33.75
Column F vs Column B	9.990	2.186	No	ns	-3.115 to 23.09
Column F vs Column C	6.969	1.525	No	ns	-6.136 to 20.07
Column F vs Column D	1.841	0.402 8	No	ns	-11.26 to 14.95
Column F vs Column E	-2.475	0.469 2	No	ns	-17.61 to 12.66

Table Analyzed	SD016				
One-way analysis of variance					
P value	0.0045				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	5.710				
R squared	0.6710				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3218	5	643.6		
Residual (within columns)	1578	14	112.7		
Total	4796	19			

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	9.391	1.083	No	ns	-15.23 to 34.01
Column F vs Column B	13.02	1.734	No	ns	-8.300 to 34.34
Column F vs Column C	-3.963	0.527 9	No	ns	-25.28 to 17.36
Column F vs Column D	-25.94	3.456	Yes	*	-47.26 to -4.620
Column F vs Column E	-16.25	2.165	No	ns	-37.57 to 5.070
Table Analyzed	SD017				
One-way analysis of variance					

P value	0.5309				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	6				
F	0.8606				
R squared	0.2351				
ANOVA Table	SS	df	MS		
Treatment (between columns)	387.8	5	77.56		
Residual (within columns)	1262	14	90.13		
Total	1650	19			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	6.943	0.8957	No	ns	-15.07 to 28.96
Column F vs Column B	9.058	1.349	No	ns	-10.01 to 28.12
Column F vs Column C	1.565	0.2331	No	ns	-17.50 to 20.63
Column F vs Column D	6.227	0.9276	No	ns	-12.84 to 25.29
Column F vs Column E	11.85	1.766	No	ns	-7.211 to 30.92

Table Analyzed	**SD018**				
One-way analysis of variance					
P value	0.0337				
P value summary	*				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	3.354				
R squared	0.5450				
ANOVA Table	SS	df	MS		
Treatment (between columns)	19280	5	3856		
Residual (within columns)	16090	14	1150		
Total	35370	19			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	-29.92	1.081	No	ns	-108.5 to 48.71
Column F vs Column B	-61.03	2.545	No	ns	-129.1 to 7.065
Column F vs Column C	-60.25	2.513	No	ns	-128.3 to 7.844
Column F vs Column D	-71.07	2.964	Yes	*	-139.2 to -2.976
Column F vs Column E	-77.72	3.242	Yes	*	-145.8 to -9.625

Table 7.4: Absolute data from MTS assay of Jurkat cells treated with 0-200 μM lignan compounds SD001-18 for 48 hours.

SD001	Metabolic Activity (%)			SD002	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	76.94346	3.174072	3	200.000	49.11661	9.369785	2
100.000	116.0483	9.617363	3	100.000	92.2556	9.589722	3
50.000	116.9317	8.939445	3	50.000	103.7691	0.8015671	3
25.000	121.5842	1.789452	3	25.000	102.2968	6.028465	3
12.500	106.861	5.209267	3	12.500	107.9211	8.751526	3
0.000	99.98528	8.936577	6	0.000	99.98528	8.936577	6

SD003	Metabolic Activity (%)			SD004	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	66.41064	5.778442	3	200.000	63.28725	6.116974	3
100.000	95.72453	6.189507	3	100.000	85.43267	15.49868	3
50.000	94.06042	6.216141	3	50.000	84.71581	2.580688	3
25.000	94.44445	0.4229965	3	25.000	93.54839	4.856972	3
12.500	99.64158	3.314499	3	12.500	96.56938	9.042885	3
0.000	100.000	8.618073	5	0.000	100.000	8.618073	5

SD005	Metabolic Activity (%)			SD006	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	60.54422	2.287659	3	200.000	37.68708	12.72064	3
100.000	82.80274	4.575302	3	100.000	87.34694	11.10565	3
50.000	94.06802	4.284241	3	50.000	107.1293	21.01725	3
25.000	92.62585	3.518105	3	25.000	97.25171	5.769028	3
12.500	96.57143	9.065636	3	12.500	97.68709	3.53418	3
0.000	60.54422	2.287659	3	0.000	100.0408	17.41274	6

SD007	Metabolic Activity (%)			SD008	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	-7.574363	1.380966	3	200.000	59.12297	10.29633	3
100.000	64.09076	1.583656	3	100.000	52.00859	7.736633	3
50.000	92.4563	10.65093	3	50.000	68.4146	10.13853	3
25.000	115.3327	7.343781	3	25.000	98.37474	6.769287	3
12.500	120.0552	15.06635	3	12.500	108.7703	6.573929	3
0.000	100.0153	7.423683	6	0.000	100.0153	7.423683	6

SD010	Metabolic Activity (%)			SD011	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	70.14323	0.3165131	2	200.000	57.37492	0.245285	3
100.000	71.82929	6.000435	3	100.000	105.1657	11.20348	3
50.000	86.69053	7.173958	3	50.000	108.0897	7.386215	3
25.000	106.3563	7.841026	3	25.000	107.050	2.583008	3
12.500	95.85197	6.839386	3	12.500	101.5919	4.964378	3
0.000	100.000	6.034309	6	0.000	99.98376	15.6543	6

SD012	Metabolic Activity (%)			SD013	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n	(uM)	Mean	Standard Deviation	n
200.000	74.48522	4.684509	2	200.000	72.41714	5.099998	2
100.000	68.27811	7.614059	3	100.000	65.65952	8.289368	3
50.000	86.60101	1.793488	3	50.000	85.60754	1.95256	3
25.000	99.07488	1.809074	3	25.000	99.18777	1.969543	3
12.500	98.98537	7.108887	3	12.500	99.09032	7.739403	3
0.000	100.000	6.034309	6	0.000	99.98376	15.6543	6

SD014	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	46.80232	5.657627	3
100.000	83.78552	2.167465	3
50.000	101.938	4.609604	3
25.000	114.6318	17.51064	3
12.500	98.90181	8.790276	3
0.000	100.000	15.4689	4

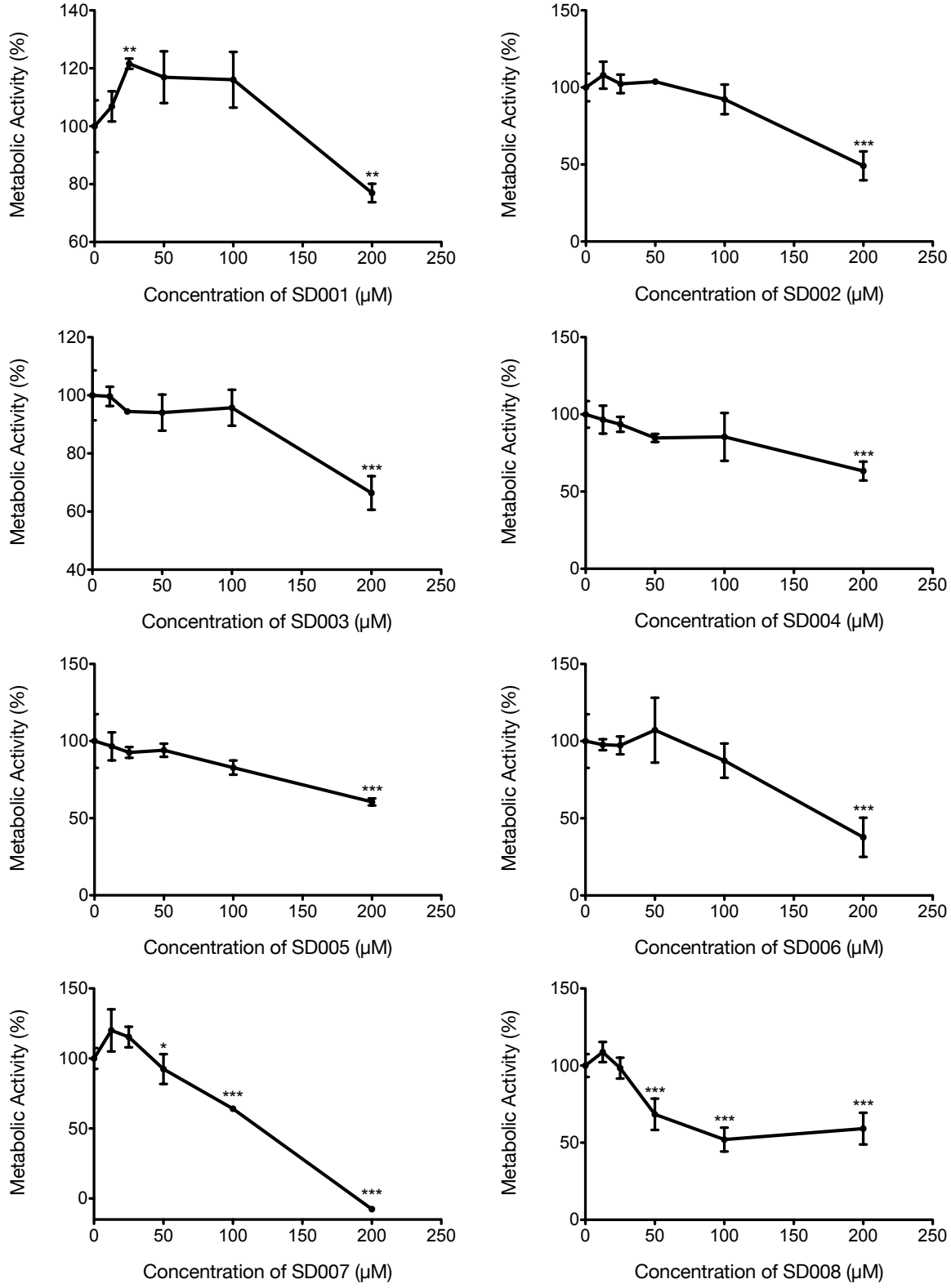
SD015	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	80.52325	5.024765	3
100.000	96.83461	15.85726	3
50.000	102.2287	5.324173	3
25.000	116.1822	19.76269	3
12.500	109.3346	8.736702	3
0.000	100.000	15.4689	4

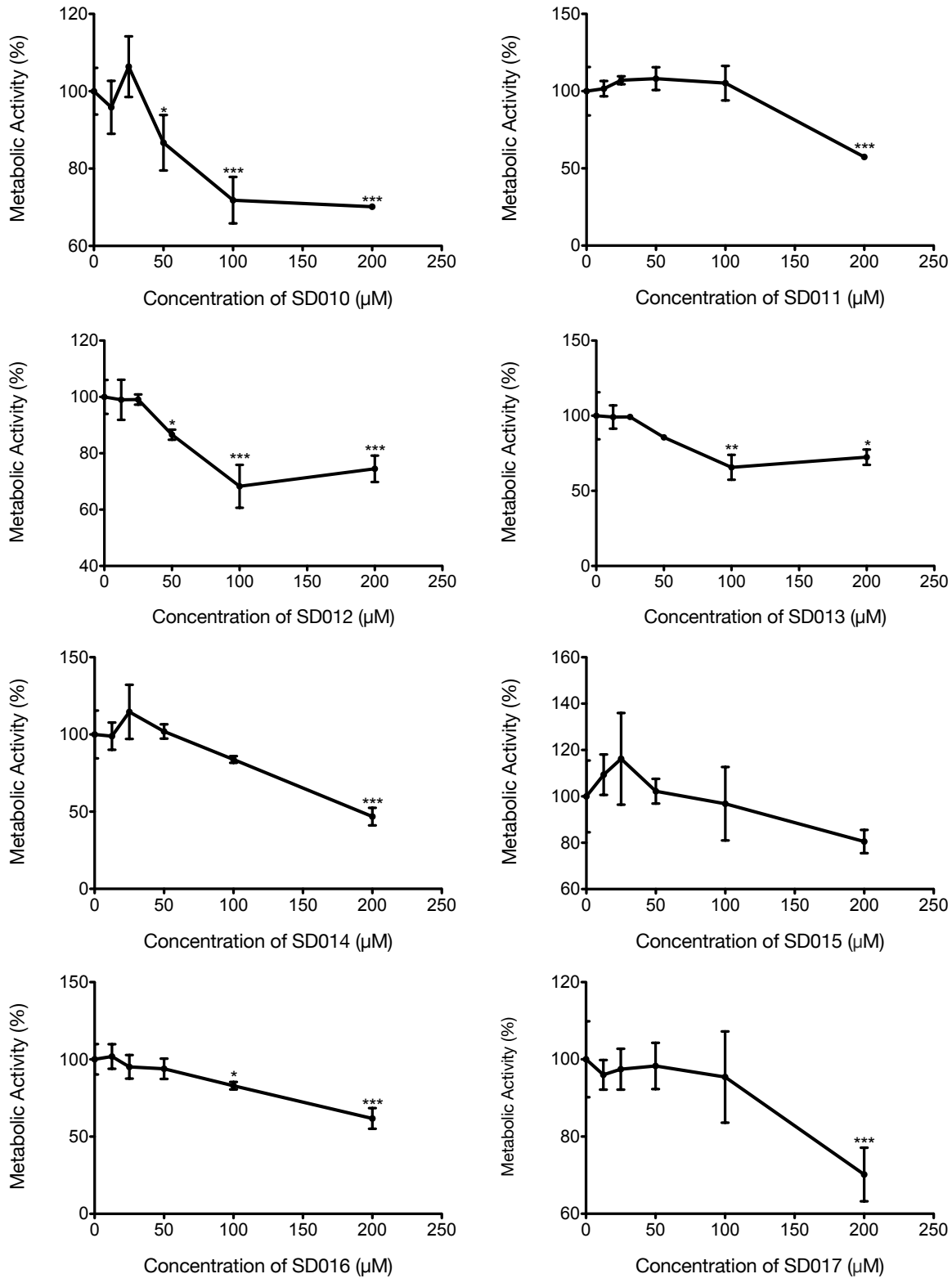
SD016	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	61.74535	6.677834	3
100.000	82.94707	2.400879	3
50.000	93.93419	6.587303	3
25.000	95.16451	7.625648	3
12.500	101.8598	7.960793	3
0.000	100.0286	9.837318	6

SD017	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	70.15737	6.942886	3
100.000	95.42204	11.81695	3
50.000	98.28326	5.979088	3
25.000	97.45351	5.286003	3
12.500	95.99429	3.840019	3
0.000	100.0286	9.837318	6

SD018	Metabolic Activity (%)		
(uM)	Mean	Standard Deviation	n
200.000	73.42902	3.403976	3
100.000	79.52153	6.144547	3
50.000	104.2105	14.10014	3
25.000	101.1483	4.306221	3
12.500	91.96171	3.729599	3
0.000	100.000	17.70909	6

Table 7.5: ***Graphs from 48-hour MTS Assays for Lignan Compounds SD001-18.*** *Data is represented as mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with Dunnett's post-hoc test used to compare samples with the untreated control. * represents a p value < 0.05, ** represents a p value < 0.01, *** represents a p value < 0.001.*




Metabolic Activity (%)
Concentration of SD010 (μM)
Concentration of SD011 (μM)
Concentration of SD012 (μM)
Concentration of SD013 (μM)
Concentration of SD014 (μM)
Concentration of SD015 (μM)
Concentration of SD016 (μM)
Concentration of SD017 (μM)

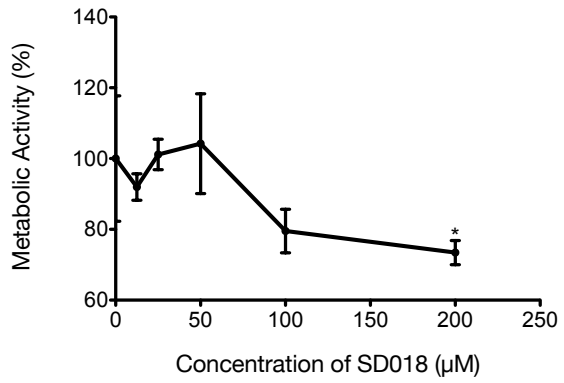

Metabolic Activity (%)
140
120
100
80
60
0
50
100
150
200
250
*
Concentration of SD018 (μM)

Table 7.6: *One-Way ANOVAs with Dunnett's post hoc test for* absolute data from MTS assay of Jurkat cells treated with 0-200 µM lignan compounds SD001-18 for 48 hours.

Table Analyzed	SD001				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	15.04				
R squared	0.8337				
ANOVA Table	SS	df	MS		
Treatment (between columns)	4136	5	827.3		
Residual (within columns)	825.0	15	55.00		
Total	4961	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	23.04	4.394	Yes	**	8.274 to 37.81
Column F vs Column B	-16.06	3.063	Yes	*	-30.83 to -1.295
Column F vs Column C	-16.95	3.232	Yes	*	-31.71 to -2.179
Column F vs Column D	-21.60	4.119	Yes	**	-36.37 to -6.831
Column F vs Column E	-6.876	1.311	No	ns	-21.64 to 7.892

Table Analyzed	SD003				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	13.10				
R squared	0.8239				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2526	5	505.2		
Residual (within columns)	540.1	14	38.58		
Total	3066	19			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	33.59	7.405	Yes	***	20.71 to 46.47
Column F vs Column B	4.275	0.9426	No	ns	-8.607 to 17.16
Column F vs Column C	5.940	1.309	No	ns	-6.943 to 18.82
Column F vs Column D	5.556	1.225	No	ns	-7.327 to 18.44
Column F vs Column E	0.3584	0.07902	No	ns	-12.52 to 13.24

Table Analyzed	SD004				
One-way analysis of variance					
P value	0.0012				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	7.573				
R squared	0.7301				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2911	5	582.3		
Residual (within columns)	1076	14	76.88		
Total	3988	19			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	36.71	5.733	Yes	***	18.53 to 54.90
Column F vs Column B	14.57	2.275	No	ns	-3.619 to 32.75
Column F vs Column C	15.28	2.387	No	ns	-2.902 to 33.47
Column F vs Column D	6.452	1.008	No	ns	-11.74 to 24.64
Column F vs Column E	3.431	0.5357	No	ns	-14.76 to 21.62

Table Analyzed	SD006				
One-way analysis of variance					
P value	0.0003				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	9.847				
R squared	0.7665				
ANOVA Table	SS	df	MS		
Treatment (between columns)	10050	5	2010		
Residual (within columns)	3061	15	204.1		
Total	13110	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	62.35	6.173	Yes	***	33.91 to 90.80
Column F vs Column B	12.69	1.257	No	ns	-15.75 to 41.14
Column F vs Column C	-7.088	0.701 7	No	ns	-35.54 to 21.36
Column F vs Column D	2.789	0.276 1	No	ns	-25.66 to 31.24
Column F vs Column E	2.354	0.233 0	No	ns	-26.09 to 30.80

Table Analyzed	SD007				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	97.68				
R squared	0.9702				
ANOVA Table	SS	df	MS		
Treatment (between columns)	34940	5	6988		
Residual (within columns)	1073	15	71.54		
Total	36010	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	107.6	17.99	Yes	***	90.75 to 124.4
Column F vs Column B	35.92	6.007	Yes	***	19.08 to 52.77
Column F vs Column C	7.559	1.264	No	ns	-9.284 to 24.40
Column F vs Column D	-15.32	2.561	No	ns	-32.16 to 1.526
Column F vs Column E	-20.04	3.351	Yes	*	-36.88 to -3.197

Table Analyzed	SD008				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	29.23				
R squared	0.9069				
ANOVA Table	SS	df	MS		
Treatment (between columns)	9655	5	1931		
Residual (within columns)	991.0	15	66.06		
Total	10650	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	40.89	7.115	Yes	***	24.71 to 57.08
Column F vs Column B	48.01	8.353	Yes	***	31.82 to 64.19
Column F vs Column C	31.60	5.498	Yes	***	15.42 to 47.79
Column F vs Column D	1.641	0.285	No	ns	-14.55 to 17.83
Column F vs Column E	-8.755	1.523	No	ns	-24.94 to 7.431

Table Analyzed	SD010
One-way analysis of variance	
P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	16.07
R squared	0.8516

ANOVA Table	SS	df	MS
Treatment (between columns)	3292	5	658.4
Residual (within columns)	573.6	14	40.97
Total	3866	19	

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	29.86	5.713	Yes	***	15.01 to 44.70
Column F vs Column B	28.17	6.224	Yes	***	15.32 to 41.03
Column F vs Column C	13.31	2.941	Yes	*	0.4546 to 26.16
Column F vs Column D	-6.356	1.404	No	ns	-19.21 to 6.499
Column F vs Column E	4.148	0.916 4	No	ns	-8.707 to 17.00

Table Analyzed	SD011
One-way analysis of variance	
P value	0.0002
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	10.37
R squared	0.7757

ANOVA Table	SS	df	MS
Treatment (between columns)	5699	5	1140
Residual (within columns)	1648	15	109.9
Total	7347	20	

Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	42.61	5.749	Yes	***	21.73 to 63.48
Column F vs Column B	-5.182	0.699 1	No	ns	-26.06 to 15.69
Column F vs Column C	-8.106	1.094	No	ns	-28.98 to 12.77
Column F vs Column D	-7.066	0.953 3	No	ns	-27.94 to 13.81
Column F vs Column E	-1.608	0.217 0	No	ns	-22.48 to 19.27

Table Analyzed	SD012				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	19.47				
R squared	0.8743				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3018	5	603.6		
Residual (within columns)	434.0	14	31.00		
Total	3452	19			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	25.51	5.612	Yes	***	12.60 to 38.43
Column F vs Column B	31.72	8.057	Yes	***	20.54 to 42.90
Column F vs Column C	13.40	3.403	Yes	*	2.217 to 24.58
Column F vs Column D	0.9251	0.2350	No	ns	-10.26 to 12.11
Column F vs Column E	1.015	0.2577	No	ns	-10.17 to 12.20

Table Analyzed	SD014				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	13.89				
R squared	0.8424				
ANOVA Table	SS	df	MS		
Treatment (between columns)	8558	5	1712		
Residual (within columns)	1602	13	123.2		
Total	10160	18			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	53.20	6.275	Yes	***	28.88 to 77.51
Column F vs Column B	16.21	1.913	No	ns	-8.099 to 40.53
Column F vs Column C	-1.938	0.2286	No	ns	-26.25 to 22.38
Column F vs Column D	-14.63	1.726	No	ns	-38.95 to 9.682
Column F vs Column E	1.098	0.1295	No	ns	-23.22 to 25.41

Table Analyzed	SD015				
One-way analysis of variance					
P value	0.0808				
P value summary	ns				
Are means signif. different? (P < 0.05)	No				
Number of groups	6				
F	2.549				
R squared	0.4951				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2218	5	443.5		
Residual (within columns)	2262	13	174.0		
Total	4479	18			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	19.48	1.933	No	ns	-9.417 to 48.37
Column F vs Column B	3.165	0.314 2	No	ns	-25.73 to 32.06
Column F vs Column C	-2.229	0.221 2	No	ns	-31.12 to 26.66
Column F vs Column D	-16.18	1.606	No	ns	-45.08 to 12.71
Column F vs Column E	-9.335	0.926 6	No	ns	-38.23 to 19.56

Table Analyzed	SD016				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	12.08				
R squared	0.8011				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3682	5	736.4		
Residual (within columns)	914.4	15	60.96		
Total	4597	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	38.28	6.934	Yes	***	22.74 to 53.83
Column F vs Column B	17.08	3.094	Yes	*	1.534 to 32.63
Column F vs Column C	6.094	1.104	No	ns	-9.454 to 21.64
Column F vs Column D	4.864	0.881 0	No	ns	-10.68 to 20.41
Column F vs Column E	-1.831	0.331 7	No	ns	-17.38 to 13.72

Table Analyzed	SD017				
One-way analysis of variance					
P value	0.0031				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	5.998				
R squared	0.6666				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2032	5	406.4		
Residual (within columns)	1016	15	67.76		
Total	3049	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	29.87	5.132	Yes	***	13.48 to 46.26
Column F vs Column B	4.607	0.791	No	ns	-11.79 to 21.00
Column F vs Column C	1.745	0.299	No	ns	-14.65 to 18.14
Column F vs Column D	2.575	0.442	No	ns	-13.82 to 18.97
Column F vs Column E	4.034	0.693	No	ns	-12.36 to 20.43
Table Analyzed	SD018				
One-way analysis of variance					
P value	0.0240				
P value summary	*				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	6				
F	3.617				
R squared	0.5466				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2567	5	513.5		
Residual (within columns)	2129	15	142.0		
Total	4697	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Column F vs Column A	26.57	3.154	Yes	*	2.845 to 50.30
Column F vs Column B	20.48	2.431	No	ns	-3.247 to 44.20
Column F vs Column C	-4.211	0.499	No	ns	-27.94 to 19.52
Column F vs Column D	-1.148	0.136	No	ns	-24.87 to 22.58
Column F vs Column E	8.038	0.954	No	ns	-15.69 to 31.76

Table 7.7: Absolute data for Annexin V/PI analysis of Jurkat cells treated for 24 hours with 0-200 µM of lignan compounds SD001-18.

Lignan Concentration (µM)	Viable			Early Apoptotic			Late Apoptotic			Necrotic		
SD001	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	73.38333	3.792049	3	18.44667	2.646136	3	7.096667	1.710624	3	1.073333	0.5205126	3
100	83.43667	6.353742	3	8.16	2.65684	3	6.593333	3.819232	3	1.81	0.362905	3
50	86.40333	0.9392726	3	6.193333	0.3150133	3	5.266667	0.3312601	3	2.136667	0.6909655	3
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD002	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	68.27334	2.410236	3	22.47	1.516541	3	8.47	1.358418	3	0.7866666	0.2702468	3
100	85.57999	1.694342	3	6.78	0.3874274	3	5.84	1.353477	3	1.8	0.506853	3
50	84.83666	2.984698	3	5.853333	1.356515	3	6.113333	1.622848	3	3.196667	1.117915	3
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD003	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	74.14667	2.231601	3	17.66667	1.985682	3	6.693333	0.4072263	3	1.493333	0.3827967	3
100	80.93	1.922418	3	11.56	1.138727	3	6.256667	0.3003888	3	1.253333	0.4895236	3
50	83.85667	0.4992336	3	9.463333	0.2458323	3	5.133333	0.3362044	3	1.546667	0.5089532	3
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD004	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	68.13667	3.988203	3	16.99	2.447183	3	13.8	2.099309	3	1.073333	0.627402	3
100	78.22334	2.633484	3	9.526668	0.5953429	3	11.48333	2.301311	3	0.7666667	0.3837099	3
50	76.00333	1.696622	3	9.75	1.305641	3	13.18333	1.956459	3	1.063333	1.342473	3
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD005	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	64.29333	3.762213	3	17.81	4.981335	3	11.16333	3.609326	3	6.733334	4.64696	3

100	83.64333	4.685105	3	6.736666	1.906471	3	4.476667	0.8800758	3	5.143333	2.027248	3
50	82.38667	2.16853	3	6.533333	1.277863	3	5.356667	1.860573	3	5.723333	4.117734	3
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD006	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	72.53333	1.459361	3	12.69667	1.182638	3	13.22	0.2685148	3	1.55	0.5086256	3
100	79.19	1.105305	3	8.146667	0.6226825	3	10.88	0.2306515	3	1.783333	0.3231615	3
50	78.80334	2.345045	3	8.150001	0.6165225	3	11.19333	1.500011	3	1.853333	0.6027714	3
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD007	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	5.8	2.041176	7	26.91333	5.79126	9	62.78778	12.95376	9	1.178889	1.720991	9
100	72.35222	2.745499	9	10.04111	0.9930062	9	14.02222	3.708699	9	3.584444	3.967664	9
50	75.90111	3.697271	9	10.64222	3.461274	9	10.58222	4.595783	9	3.232222	3.672488	9
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD008	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	49.79667	5.208803	6	28.45	3.685567	6	20.645	1.66583	6	1.108333	0.9087666	6
100	73.90833	4.535635	6	11.83	2.161657	6	12.905	2.234697	6	1.356667	0.9609092	6
50	73.865	7.82549	6	11.96667	3.21561	6	12.45333	3.410922	6	1.715	1.484503	6
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD009	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	46.52	8.027529	6	35.15333	7.588181	6	17.67333	1.09469	6	0.6533334	0.5246586	6
100	72.64167	3.654972	6	13.17833	2.169843	6	12.78667	1.478738	6	1.393333	1.03857	6
50	72.83833	5.03945	6	13.15333	2.738275	6	12.46833	1.612469	6	1.54	1.058603	6
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD010	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	46.28222	23.25723	9	24.83	11.82619	9	27.93111	11.19931	9	0.9566666	0.5727565	9
100	62.07889	17.7979	9	17.01	7.480533	9	19.59222	9.902913	9	1.318889	0.7513229	9
50	70.65667	6.15797	9	14.37667	3.563208	9	13.49556	3.421649	9	1.47	1.263576	9

0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD011	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	76.31	0.806164	3	12.16333	0.2173322	3	10.59667	0.8470145	3	0.93	0.23	3
100	76.19666	0.5021289	3	11.67667	0.3787259	3	9.673333	0.1011601	3	2.453333	0.4359281	3
50	75.82667	1.529585	3	10.64667	0.4119869	3	10.52333	1.092261	3	3.003333	0.458294	3
0	79.11445	6.406799	18	7.427778	1.728895	18	10.92222	5.280658	18	2.535556	2.203968	18
SD012	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	35.90333	2.742414	3	13.67	1.70845	3	49.04667	1.570552	3	1.38	0.7211103	3
100	65.66334	2.952495	3	8.133333	0.6074814	3	24.09333	1.783041	3	2.11	0.5726256	3
50	72.61333	1.369538	3	7.146667	0.4536887	3	18.95667	1.255242	3	1.283333	0.1778576	3
0	85.54	2.984364	9	5.208889	0.6466538	9	8.147778	2.224583	9	1.103333	0.3783186	9
SD013	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	34.42667	1.211295	3	16.37	0.2511974	3	44.19333	0.8500775	3	5.01	0.3143246	3
100	41.20333	2.815286	3	15.60667	0.9392726	3	40.67667	0.7392116	3	2.513333	1.68693	3
50	74.88	2.013429	3	11.72333	1.519517	3	12.78333	0.3622615	3	0.6133333	0.136504	3
0	85.54	2.984364	9	5.208889	0.6466538	9	8.147778	2.224583	9	1.103333	0.3783186	9
SD014	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	55.04	2.934552	3	7.28	0.2718456	3	34.65667	3.320426	3	3.023333	1.098651	3
100	72.32999	1.203787	3	11.5	1.300461	3	14.64333	0.1887683	3	1.526667	0.340196	3
50	75.92333	4.584337	3	12.41667	0.6900966	3	10.45667	3.308812	3	1.203333	0.6607824	3
0	85.54	2.984364	9	5.208889	0.6466538	9	8.147778	2.224583	9	1.103333	0.3783186	9
SD015	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	75.96	1.67699	3	8.7	0.6295236	3	14.58333	1.341243	3	0.7566667	0.211266	3
100	76.42333	1.535752	3	12.15333	0.9667127	3	10.59333	0.7484872	3	0.83	0.2523886	3
50	79.78333	2.371691	3	11.31667	1.297318	3	8.36	1.040577	3	0.54	0.1374773	3
0	85.54	2.984364	9	5.208889	0.6466538	9	8.147778	2.224583	9	1.103333	0.3783186	9
SD016	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n

200	67.20667	1.151532	3	13.52333	0.7240396	3	17.91333	1.224595	3	1.356667	0.4476978	3
100	69.87	2.743375	3	18.65667	2.587128	3	10	1.213095	3	1.473333	0.4697162	3
50	74.88	1.729886	3	17.21333	1.819954	3	7.043334	0.4100405	3	0.8633333	0.4554485	3
0	85.54	2.984364	9	5.208889	0.6466538	9	8.147778	2.224583	9	1.103333	0.3783186	9
SD017	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	84.53666	2.105049	3	8.473333	0.9700685	3	6.336667	0.9411873	3	0.6533333	0.2773686	3
100	83.66333	3.324222	3	7.91	0.8601745	3	7.28	2.098666	3	1.146667	0.3693689	3
50	87.48334	2.094047	3	5.4	0.1915726	3	6.003334	1.521326	3	1.113333	0.6035175	3
0	85.54	2.984364	9	5.208889	0.6466538	9	8.147778	2.224583	9	1.103333	0.3783186	9
SD018	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
200	37.40333	1.713164	3	19.73667	0.5231001	3	40.46333	0.6724831	3	2.396667	0.6789208	3
100	47.15	2.990535	3	18.16667	0.4473628	3	32.94667	1.903927	3	1.736667	0.6782576	3
50	67.79	5.430689	3	23.26	5.420212	3	8.523334	0.1365042	3	0.4266666	0.1527525	3
0	85.54	2.984364	9	5.208889	0.6466538	9	8.147778	2.224583	9	1.103333	0.3783186	9

Table 7.8: Cell cycle *analysis for Jurkat cells in G_1-phase following 4 hours incubation with 100 µM lignan compounds SD001-18 and camptothecin.*

	Control	SD001	SD002	SD003	SD004	SD006	SD007	SD008	SD010	SD011	SD012	SD013	SD014	SD015	SD016	SD017	SD018	Pos. Control
Number of values	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Minimum	64.51	65.81	64.83	64.98	64.68	64.76	65.31	66.21	66.16	65.67	67.13	66.65	66.51	65.71	65.73	66.16	65.77	76.4
25% Percentile	64.51	65.81	64.83	64.98	64.68	64.76	65.31	66.21	66.16	65.67	67.13	66.65	66.51	65.71	65.73	66.16	65.77	76.4
Median	65.96	65.93	65.23	65.3	65.52	65.31	67.04	67.1	66.78	66.29	67.24	67.22	66.54	66.63	66.15	66.84	66.25	76.85
75% Percentile	67.29	66.65	65.9	66.53	66.75	67.39	67.94	67.99	67.87	67.31	67.96	67.35	66.95	66.75	68.86	67.47	69.61	79.06
Maximum	67.29	66.65	65.9	66.53	66.75	67.39	67.94	67.99	67.87	67.31	67.96	67.35	66.95	66.75	68.86	67.47	69.61	79.06
Mean	65.92	66.13	65.32	65.6	65.65	65.82	66.76	67.1	66.94	66.42	67.44	67.07	66.67	66.36	66.91	66.82	67.21	77.44
Std. Deviation	1.39	0.4543	0.5406	0.8183	1.041	1.387	1.337	0.89	0.8657	0.8281	0.4508	0.3723	0.2458	0.569	1.699	0.6552	2.092	1.424
Std. Error	0.8028	0.2623	0.3121	0.4725	0.6011	0.8009	0.7717	0.5138	0.4998	0.4781	0.2603	0.215	0.1419	0.3285	0.9809	0.3783	1.208	0.822
Lower 95% CI of mean	62.47	65	63.98	63.57	63.06	62.37	63.44	64.89	64.79	64.37	66.32	66.15	66.06	64.95	62.69	65.2	62.01	73.9
Upper 95% CI of mean	69.37	67.26	66.66	67.64	68.24	69.27	70.08	69.31	69.09	68.48	68.56	68	67.28	67.78	71.13	68.45	72.41	80.97
Sum	197.8	198.4	196	196.8	197	197.5	200.3	201.3	200.8	199.3	202.3	201.2	200	199.1	200.7	200.5	201.6	232.3

Table 7.9: *Cell cycle analysis for Jurkat cells in S-phase following 4 hours incubation with 100 µM lignan compounds SD001-18 and camptothecin.*

	Control	SD001	SD002	SD003	SD004	SD006	SD007	SD008	SD010	SD011	SD012	SD013	SD014	SD015	SD016	SD017	SD018	Pos. Control
Number of values	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Minimum	23.64	24.05	24.6	24.04	24.19	23.52	24.74	25.69	25.81	24.3	25.4	25.39	25.81	25.96	24.58	22.01	24.21	16.59
25% Percentile	23.64	24.05	24.6	24.04	24.19	23.52	24.74	25.69	25.81	24.3	25.4	25.39	25.81	25.96	24.58	22.01	24.21	16.59
Median	24.17	24.54	25.63	25.16	25.36	25.03	25.33	26.54	26.72	25.13	25.79	26.32	25.84	26.7	27.86	24.7	26.55	17.4
75% Percentile	25.6	24.56	26.2	25.85	25.53	26.23	26.83	26.67	26.82	25.68	26.11	26.56	26.54	26.85	27.97	25.5	27.6	18.32
Maximum	25.6	24.56	26.2	25.85	25.53	26.23	26.83	26.67	26.82	25.68	26.11	26.56	26.54	26.85	27.97	25.5	27.6	18.32
Mean	24.47	24.38	25.48	25.02	25.03	24.93	25.63	26.3	26.45	25.04	25.77	26.09	26.06	26.5	26.8	24.07	26.12	17.44
Std. Deviation	1.014	0.2888	0.8109	0.9135	0.7295	1.358	1.078	0.5323	0.5565	0.6947	0.3556	0.618	0.4131	0.4765	1.926	1.828	1.735	0.8656
Std. Error	0.5853	0.1668	0.4682	0.5274	0.4212	0.784	0.6221	0.3073	0.3213	0.4011	0.2053	0.3568	0.2385	0.2751	1.112	1.056	1.002	0.4997
Lower 95% CI of mean	21.95	23.67	23.46	22.75	23.21	21.55	22.96	24.98	25.07	23.31	24.88	24.55	25.04	25.32	22.02	19.53	21.81	15.29
Upper 95% CI of mean	26.99	25.1	27.49	27.29	26.84	28.3	28.31	27.62	27.83	26.76	26.65	27.63	27.09	27.69	31.59	28.61	30.43	19.59
Sum	73.41	73.15	76.43	75.05	75.08	74.78	76.9	78.9	79.35	75.11	77.3	78.27	78.19	79.51	80.41	72.21	78.36	52.31

Table 7.10: *Cell cycle analysis for Jurkat cells in G_2-phase following 4 hours incubation with 100 µM lignan compounds SD001-18 and camptothecin (positive control).*

	Control	SD001	SD002	SD003	SD004	SD006	SD007	SD008	SD010	SD011	SD012	SD013	SD014	SD015	SD016	SD017	SD018	Pos. Control
Number of values	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Minimum	9.09	9.3	8.57	9.18	8.95	9	7.32	6.23	6.32	8.39	6.64	6.47	6.92	6.55	6	8.34	6.15	4.34
25% Percentile	9.09	9.3	8.57	9.18	8.95	9	7.32	6.23	6.32	8.39	6.64	6.47	6.92	6.55	6	8.34	6.15	4.34
Median	9.87	9.5	9.5	9.44	9.11	9.09	7.63	6.32	6.5	8.58	6.65	6.78	7.24	7.41	6.3	8.46	6.18	5.28
75% Percentile	9.89	9.64	9.54	9.54	9.95	9.66	7.86	7.24	7.02	8.64	7.09	7.27	7.65	7.43	6.56	10.52	7.69	5.75
Maximum	9.89	9.64	9.54	9.54	9.95	9.66	7.86	7.24	7.02	8.64	7.09	7.27	7.65	7.43	6.56	10.52	7.69	5.75
Mean	9.617	9.48	9.203	9.387	9.337	9.25	7.603	6.597	6.613	8.537	6.793	6.84	7.27	7.13	6.287	9.107	6.673	5.123
Std. Deviation	0.4562	0.1709	0.5488	0.1858	0.5372	0.3579	0.271	0.559	0.3635	0.1305	0.257	0.4034	0.3659	0.5024	0.2802	1.225	0.8806	0.7179
Std. Error	0.2634	0.09866	0.3169	0.1073	0.3101	0.2066	0.1565	0.3227	0.2099	0.07535	0.1484	0.2329	0.2113	0.2901	0.1618	0.7075	0.5084	0.4145
Lower 95% CI of mean	8.483	9.056	7.84	8.925	8.002	8.361	6.93	5.208	5.71	8.212	6.155	5.838	6.361	5.882	5.591	6.062	4.486	3.34
Upper 95% CI of mean	10.75	9.904	10.57	9.848	10.67	10.14	8.276	7.985	7.516	8.861	7.432	7.842	8.179	8.378	6.983	12.15	8.861	6.907
Sum	28.85	28.44	27.61	28.16	28.01	27.75	22.81	19.79	19.84	25.61	20.38	20.52	21.81	21.39	18.86	27.32	20.02	15.37

Table 7.11: *One-Way ANOVA and Dunnet's Post hoc test of Jurkat cells in G_1-phase following 4 hours treatment with 100 µM lignan compounds SD001-18 and camptothecin compared to the untreated control*

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	18				
F	18.51				
R squared	0.8974				
ANOVA Table	SS	df	MS		
Treatment (between columns)	359.7	17	21.16		
Residual (within columns)	41.14	36	1.143		
Total	400.8	53			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs SD001	-0.2100	0.2406	No	ns	-2.849 to 2.429
Control vs SD002	0.6000	0.6874	No	ns	-2.039 to 3.239
Control vs SD003	0.3167	0.3628	No	ns	-2.323 to 2.956
Control vs SD004	0.2700	0.3093	No	ns	-2.369 to 2.909
Control vs SD006	0.1000	0.1146	No	ns	-2.539 to 2.739
Control vs SD007	-0.8433	0.9662	No	ns	-3.483 to 1.796
Control vs SD008	-1.180	1.352	No	ns	-3.819 to 1.459
Control vs SD010	-1.017	1.165	No	ns	-3.656 to 1.623
Control vs SD011	-0.5033	0.5767	No	ns	-3.143 to 2.136
Control vs SD012	-1.523	1.745	No	ns	-4.163 to 1.116
Control vs SD013	-1.153	1.321	No	ns	-3.793 to 1.486
Control vs SD014	-0.7467	0.8554	No	ns	-3.386 to 1.893
Control vs SD015	-0.4433	0.5079	No	ns	-3.083 to 2.196
Control vs SD016	-0.9933	1.138	No	ns	-3.633 to 1.646
Control vs SD017	-0.9033	1.035	No	ns	-3.543 to 1.736
Control vs SD018	-1.290	1.478	No	ns	-3.929 to 1.349
Control vs Camptothecin	-11.52	13.19	Yes	***	-14.16 to -8.877

Table 7.12: *One-Way ANOVA Jurkat cells in S-phase following 4 hours incubation with 100 µM lignan compounds SD001-18 and camptothecin compared to the untreated control.*

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	18				
F	12.19				
R squared	0.8520				
ANOVA Table	SS	df	MS		
Treatment (between columns)	218.1	17	12.83		
Residual (within columns)	37.87	36	1.052		
Total	256.0	53			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs SD001	0.08667	0.1035	No	ns	-2.446 to 2.619
Control vs SD002	-1.007	1.202	No	ns	-3.539 to 1.526
Control vs SD003	-0.5467	0.6528	No	ns	-3.079 to 1.986
Control vs SD004	-0.5567	0.6647	No	ns	-3.089 to 1.976
Control vs SD006	-0.4567	0.5453	No	ns	-2.989 to 2.076
Control vs SD007	-1.163	1.389	No	ns	-3.696 to 1.369
Control vs SD008	-1.830	2.185	No	ns	-4.362 to 0.7024
Control vs SD010	-1.980	2.364	No	ns	-4.512 to 0.5524
Control vs SD011	-0.5667	0.6766	No	ns	-3.099 to 1.966
Control vs SD012	-1.297	1.548	No	ns	-3.829 to 1.236
Control vs SD013	-1.620	1.934	No	ns	-4.152 to 0.9124
Control vs SD014	-1.593	1.903	No	ns	-4.126 to 0.9391
Control vs SD015	-2.033	2.428	No	ns	-4.566 to 0.4991
Control vs SD016	-2.333	2.786	No	ns	-4.866 to 0.1991
Control vs SD017	0.4000	0.4776	No	ns	-2.132 to 2.932
Control vs SD018	-1.650	1.970	No	ns	-4.182 to 0.8824
Control vs Camptothecin	7.033	8.398	Yes	***	4.501 to 9.566

Table 7.13: *One-Way ANOVA Jurkat cells in G_2-phase following 4 hours incubation with 100 µM lignan compounds SD001-18 and camptothecin compared to the untreated control.*

Table Analyzed	G2				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	18				
F	21.30				
R squared	0.9096				
ANOVA Table	SS	df	MS		
Treatment (between columns)	100.8	17	5.929		
Residual (within columns)	10.02	36	0.2784		
Total	110.8	53			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs SD001	0.1367	0.3172	No	ns	-1.166 to 1.439
Control vs SD002	0.4133	0.9595	No	ns	-0.8894 to 1.716
Control vs SD003	0.2300	0.5339	No	ns	-1.073 to 1.533
Control vs SD004	0.2800	0.6500	No	ns	-1.023 to 1.583
Control vs SD006	0.3667	0.8511	No	ns	-0.9360 to 1.669
Control vs SD007	2.013	4.673	Yes	***	0.7106 to 3.316
Control vs SD008	3.020	7.010	Yes	***	1.717 to 4.323
Control vs SD010	3.003	6.971	Yes	***	1.701 to 4.306
Control vs SD011	1.080	2.507	No	ns	-0.2227 to 2.383
Control vs SD012	2.823	6.554	Yes	***	1.521 to 4.126
Control vs SD013	2.777	6.445	Yes	***	1.474 to 4.079
Control vs SD014	2.347	5.447	Yes	***	1.044 to 3.649
Control vs SD015	2.487	5.772	Yes	***	1.184 to 3.789
Control vs SD016	3.330	7.730	Yes	***	2.027 to 4.633
Control vs SD017	0.5100	1.184	No	ns	-0.7927 to 1.813
Control vs SD018	2.943	6.832	Yes	***	1.641 to 4.246
Control vs Camptothecin	4.493	10.43	Yes	***	3.191 to 5.796

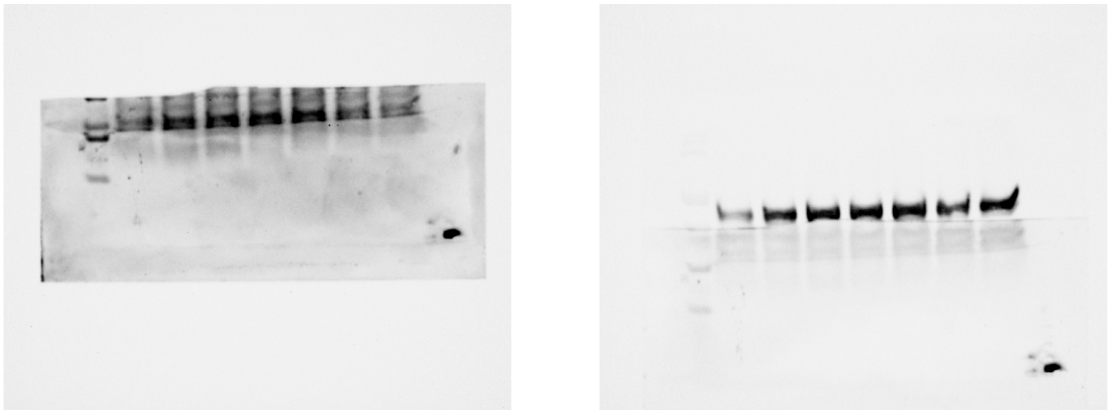


Figure 7.1: *Western Blot images of HSPB1 levels (left) and β-actin levels (right) using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

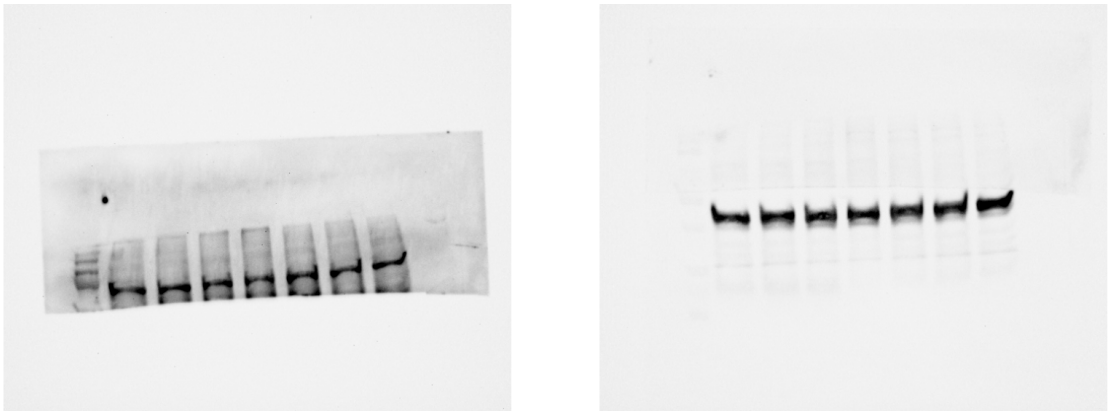


Figure 7.2: *Western Blot images of HSPA1A levels (left) and β-actin levels (right) using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

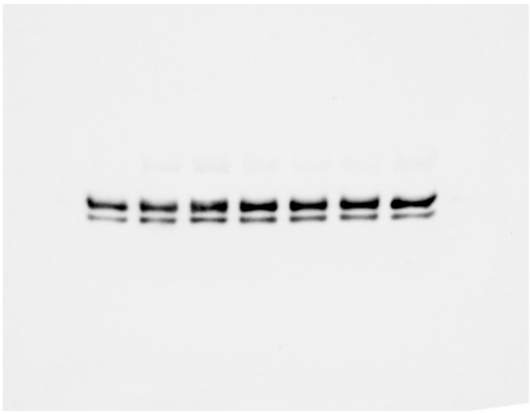


Figure 7.3: *Western Blot images of p-Akt levels and β-actin levels using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

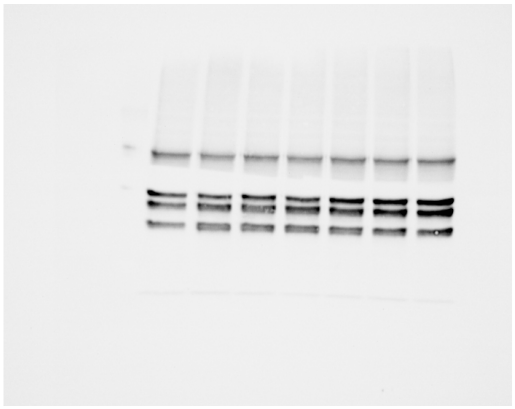


Figure 7.4: *Western Blot images of Akt levels and β-actin levels using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*

Figure 7.5: *Western Blot images of phospo-ß-catenin levels and β-actin levels using protein extracts from Jurkat cells treated for 4 hours with 100 μM of lignan compounds SD007, SD010, SD011, SD012, SD013 and SD018, and an untreated control.*